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Strategies to sensitize bladder cancer cells to small molecule inhibitors targeting the PI3K pathway

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STRATEGIES TO SENSITIZE BLADDER CANCER CELLS TO SMALL MOLECULE INHIBITORS TARGETING THE PI3K PATHWAY

by

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A

DISSERTATION

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for the Degree of

DOCTOR of PHILOSOPHY

by

Giovanni Nitti, M.S.
Houston, Texas
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DEDICATION

To my father, who has always supported me from the very beginning of my education. Thank you for your constant support and for always challenging me to improve myself as a scientist and as a person. To my mother, who is the person that I have missed the most during this training that was completed so far away from home. You and dad are the ones who I want to make proud most of all. To my grandfather Niní, who watches over me from above and still remains the person that has inspired me the most throughout my life. To my girlfriend Abril, you showed me what sacrifice is all about with your example day after day. Thank you for supporting me in my moments of weakness and for always being on my side during all these years. Thank you all for being part of my life; this thesis and my doctorate are dedicated to you.

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Thank you so much to all of those who, despite the distance, the difference in time and the phone bill, always made me feel like there was a piece of Italy right next to me here in Houston. God bless you all.

STRATEGIES TO SENSITIZE BLADDER CANCER CELLS TO SMALL MOLECULE INHIBITORS TARGETING THE PI3K PATHWAY

Giovanni Nitti, M.S.

Supervisory Professor: David J. McConkey, Ph.D.

After many years of cancer research, it is well accepted by the scientific community that the future cure for this disease lies in a personalized therapeutic approach. Anticipating therapeutic outcome based on the genetic signature of a tumor has become the new paradigm. The PI3K pathway represents an ideal target for bladder cancer, as many of the key proteins of this pathway are altered or mutated in this particular type of cancer. Several small molecule inhibitors have been developed to target this pathway, but their efficacy has been shown to be heterogeneous among different cell lines and mostly cytostatic but not cytotoxic in the case of bladder cancer. Understanding the genetic heterogeneity that underlies the efficacy of PI3K-pathway inhibitors in bladder cancer models is critical for informing future single-agent and combination therapies.

I currently have available in our laboratory three small molecule inhibitors that target the PI3K pathway at three different levels: IGF-1 receptor, AKT, and double mTOR inhibitor, which targets both TORC1 and TORC2. In this thesis, I investigate the link between sensitivity to small molecule inhibitors targeting the PI3K pathway and the genetic signature of a large panel of bladder cancer cell lines. I investigate novel strategies to sensitize bladder cancer cells to such inhibitors by targeting autophagy, perhaps one of the main resistance mechanisms used by bladder cancer cells to evade apoptosis and I outline the intricate regulation of downstream

pathways in cell lines dependent on more than one growth factor receptor. Understanding the mechanisms that bladder cancer cells use to escape currently available small molecule inhibitors may help us in designing combined approaches that are able to overcome these biological processes.

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CHAPTER 1. INTRODUCTION

1.1 Bladder Cancer

Bladder cancer represents the 5th most common cancer worldwide, responsible for about 3% of all the cancer related deaths in the United States and the 9th deadliest disease in American men (1). The incidence of this disease is 3-4 times higher in males than females (2), probably due to environmental and behavioral factors. Cigarette smoking is the most common risk factor, accounting for about 50% of all bladder cancer cases. Past studies have shown that the risk of developing Urothelial Cancer (UC) increases proportionally with the number of cigarettes smoked and that current smokers have a higher probability of developing Urothelial Cancer than former smokers (3,4). The effect of cigarette smoke on UC development is probably due to the high presence of aromatic amines and other carcinogens causing alterations in genes responsible for DNA damage repair and maintenance of homeostasis. Occupational exposure to aromatic amines has also been shown to be highly associated with bladder cancer onset. The American Cancer Society estimates that there will be about 72,570 new cases of bladder cancer and approximately 15,210 deaths in 2013, mainly affecting Caucasian men between 65 and 84 years of age (5,6). Bladder cancer has been reported to be also very common in China, showing a remarkable increase in the number of cases during the past 25 years (7) .

The development of bladder cancer depends on the alteration of five crucial processes: cell-cycle regulation, cell death, cell growth, signal transduction and gene regulation. Tumor maintenance and progression instead, relies on angiogenesis and cell invasion (Figure 1.1) (8). The perfect tuning of stochastic alterations in these mechanisms favors the formation and progression of UC throughout its four stages T1-T4. In this introduction I will provide an overview of where and how these alterations happen and what their clinical significance is for the patient as well as their therapeutic implications.

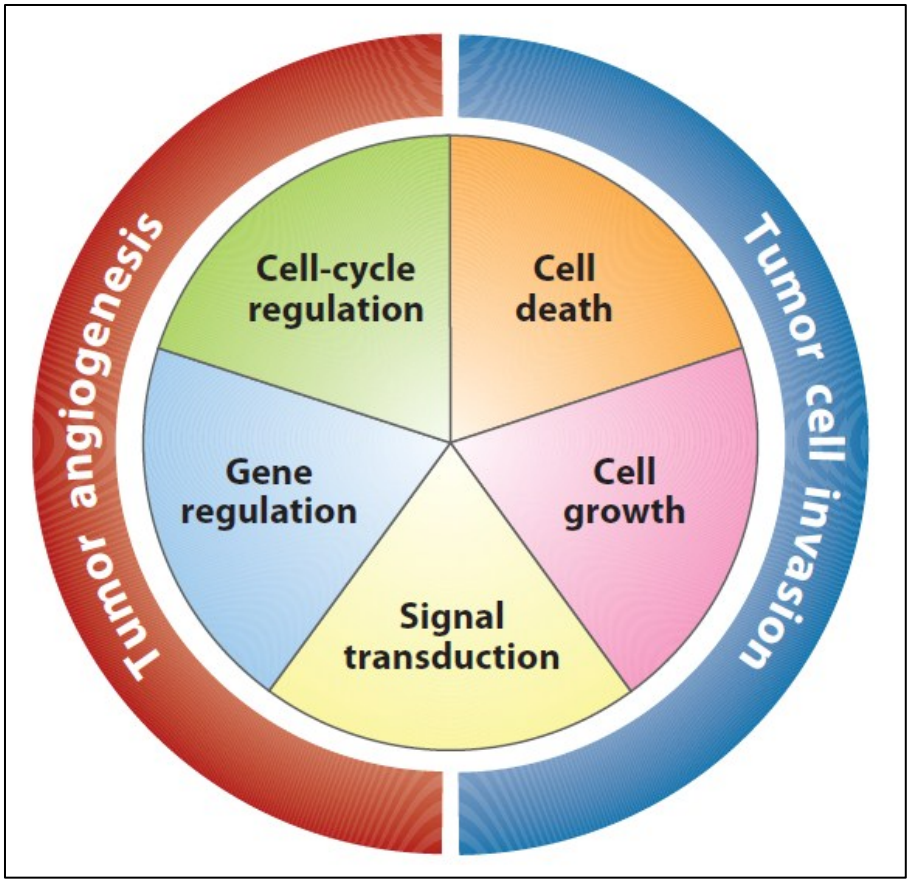


Figure1.1: Aberrant cellular processes contributing to bladder tumorigenesis. Malignant transformation of the bladder urothelium involves alterations in five intrinsic cellular processes (*central pie*) that can respond to external carcinogenic cues or that can be affected by genetic alterations. Tumor maintenance, progression, and metastasis also depend on two extrinsic processes, angiogenesis and invasion, which regulate tumor interaction with stromal elements and adjacent cells. Adapted from "Mitra, A. P., and Cote, R. J. (2009) Molecular pathogenesis and diagnostics of bladder cancer. *Annual review of pathology* **4**, 251-285" (5) with the permission of the journal.

Bladder cancer is, mainly divided into two types: muscle invasive and non-muscle invasive (Figure 1.2). Non-muscle invasive bladder cancer accounts for about 80-85% of all bladder tumors developing prevalently from papillary neo-formations. Non-muscle invasive bladder cancer can be divided into papillary carcinoma (Ta) and carcinoma *in situ* (CIS). The former is rarely lethal and easily removed surgically. Unfortunately, however, its tendency to recur makes this type of cancer a considerable economic burden for the patients (9). Intravesical chemotherapy seems to reduce recurrence in superficial bladder cancer (10). CIS instead, is the

gateway to muscle invasive bladder cancer. It derives from the non-papillary pathway, progressing from severe dysplasia, and although it represents only 15-20% of the cases of bladder cancer; it poses a potential life threatening situation for the patients (Figure1.3).

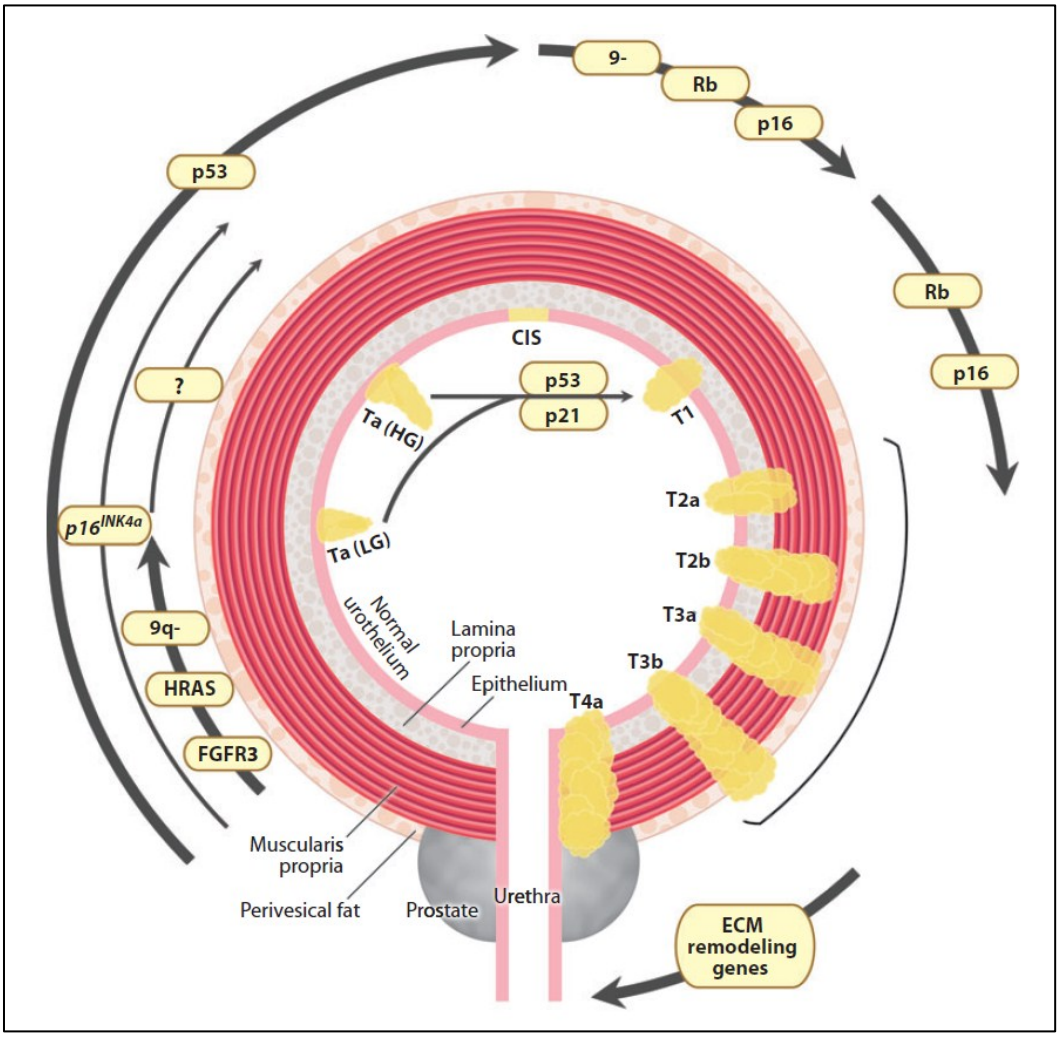


Figure 1.2 Composite model for urothelial tumorigenesis and progression**.** Noninvasive and invasive tumors are characterized by distinct molecular alterations. Although noninvasive tumors have constitutive activation of the Ras-MAPK (mitogen-activated protein kinase) pathway, flat carcinoma in situ (CIS) and invasive lesions have alterations in p53 and other cell-cycle-regulatory molecules. Loss of heterozygosity of 9q is more common in low-grade papillary carcinomas (Ta), although deletions of chromosome 9 are also seen in progressive CIS. Locations of molecules indicate characteristic alterations that pose a risk for the progression of a particular phenotype. The thickness of the arrows is approximately proportionate to the relative frequency of occurrence. Locations of the arrow tails and heads correspond to the tumor stage(s) before and after alteration(s) of the denoted molecule(s), respectively. Abbreviations: ECM, extracellular matrix; FGFR3, fibroblast growth factor receptor 3; HG, high-grade; HRAS, protein of the Harvey rat sarcoma viral oncogene homolog gene; LG, low-grade; Rb, retinoblastoma protein. Adapted from "Mitra, A. P., and Cote, R. J. (2009) Molecular pathogenesis and diagnostics of bladder cancer. *Annual review of pathology* **4**, 251-285" (5) with the permission of the journal.

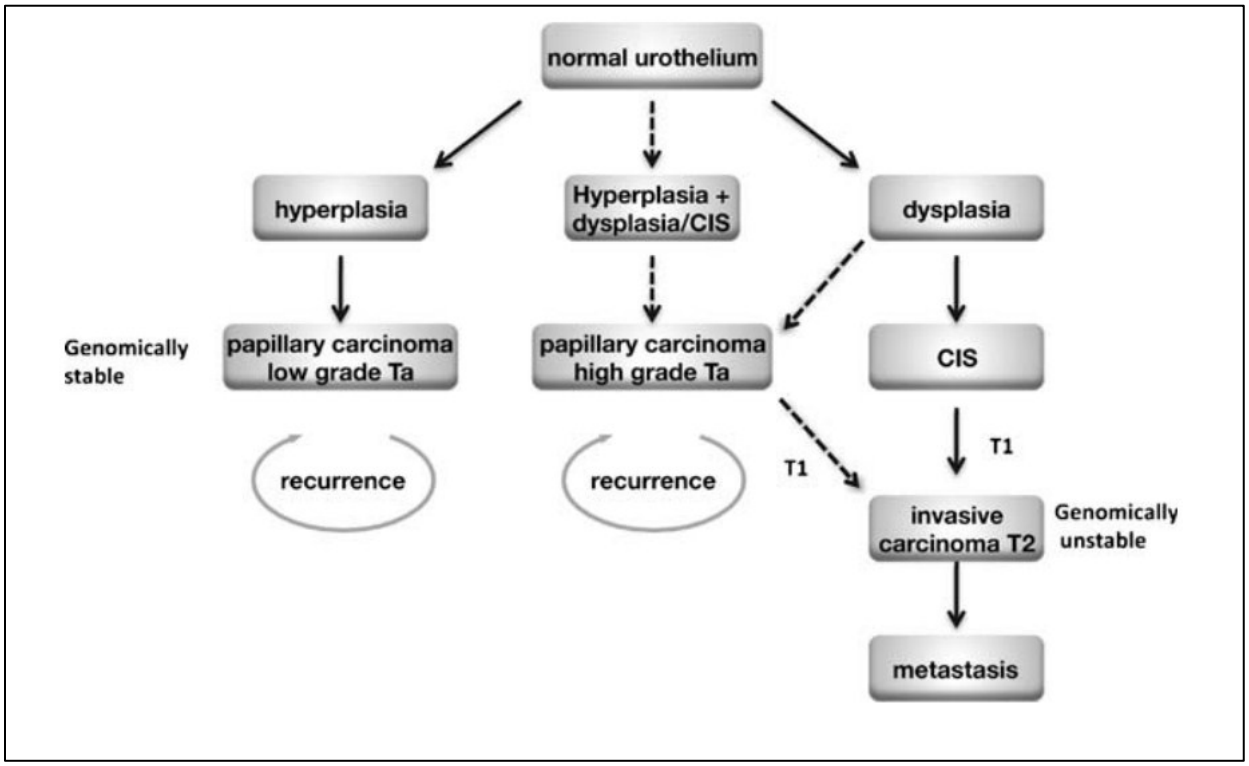


Figure1.3: Potential pathways of urothelial tumorigenesis. Solid arrows indicate likely pathways and broken arrows indicate uncertain relationships. Low-grade papillary tumors (left) may arise via simple hyperplasia and minimal dysplasia. Invasive carcinoma (right) is believed to arise via the flat high-grade lesion CIS. A third hypothetical pathway to the development of high grade papillary tumors is shown (middle). The pathway to development of T1 tumors is uncertain. Adapted from "Knowles, M. A., Platt, F. M., Ross, R. L., and Hurst, C. D. (2009) Phosphatidylinositol 3-kinase (PI3K) pathway activation in bladder cancer. *Cancer Metastasis Rev* **28**, 305-316" (11) with the permission of the journal.

Low-grade tumors have been shown to harbor H-RAS and FGFR3 mutations more often than invasive bladder cancer. FGFR3 mutations in particular have been reported altered in about 70% of Ta cancer versus 10-20% of the invasive UC cases (12-14). High-grade tumors are instead characterized by the predominant alterations in p53, Rb, p21 and p16 (Figure 1.4c) (15-18). Moreover, a strong link between the number of chromosomal aberrations and tumor stage has been reported (19) (Figure 1.4b). Tumor grade can also be influenced by behavioral habits like the number of cigarettes smoked per day and whether or not the patient is a current or an ex-smoker (20) (figure 1.4a).

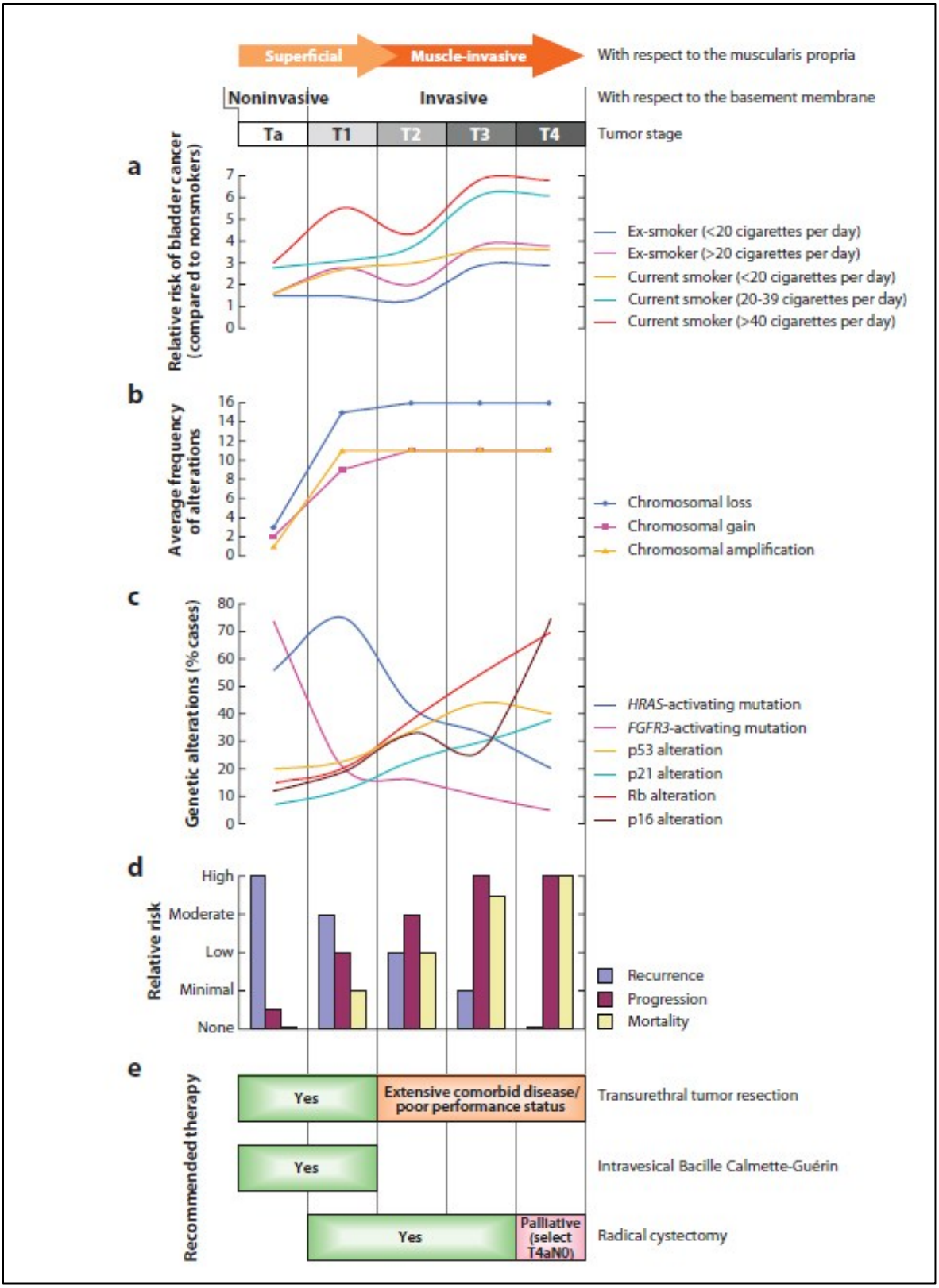


Figure1.4: Distinctive features of bladder cancer in different tumor stages. Adapted from "Mitra, A. P., and Cote, R. J. (2009) Molecular pathogenesis and diagnostics of bladder cancer. *Annual review of pathology* **4**, 251-285" (5) with the permission of the journal.

Although the majority of non-muscle invasive bladder cancer, if diagnosed early, translates into good prognosis, patients affected by muscle invasive cancer are associated with a significantly reduced 5-year survival rate (21,22). Treatments for localized muscle invasive bladder cancer still have a 5-year survival of about 80-85% after a radical cystectomy and lymphadenectomy (23) (Figure 1.4d). However, in patients with locally advanced disease, surgery alone is usually ineffective and needs to be associated with multiagent chemotherapy (24). Finally, metastatic disease represents the most difficult stage to treat with a median survival rate of 13-18 months and 10-15% disease free survival rate. The combination of methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) is the current therapy of this type of advanced disease and it has been used with very modest success during the past 30 years (25). The lack of options for the treatment of advanced and unresectable metastatic disease, along with the complete absence of an effective targeted therapy for bladder cancer raises the need for improved and more effective therapeutic approaches. One of the scopes of this thesis is to find novel strategies to improve the effects of pre-existing drugs for the treatment of bladder cancer in an effort to open new therapeutic horizons for the treatment of this type of cancer.

1.1.1 Major alterations in bladder cancer

Just like many other types of cancer, UC also carries its own unique genetic signature. Many genes have been reported to be mutated in bladder cancer. Genes regulating cell cycle are often altered in UC and p53 is perhaps the most important of these. p53 is a tumor suppressor gene located on chromosome 17 that regulates DNA repair, mediates cell cycle arrest in G1/S phase to allow DNA damage repair, and promotes apoptosis in the case of unrepairable damage (26). In physiological conditions, the half-life of p53 is usually very short but in UC, it has been found

that mutated p53 escapes degradation with consequential accumulation into the nucleus (27). This nuclear accumulation represents one of the distinctive features of p53 mutated cancers. Because of its role in regulating such crucial cell processes, it is not surprising that p53 mutations are the most common among the mutations that occur in UC. Bladder cancers harboring p53 mutations are also prone to be very aggressive with a tendency to progress towards the most advanced stages of this disease. The presence of altered p53, as well as Rb, is indeed considered to be a signature feature of muscle invasive disease, while the frequency of mutations in these two genes is very low in non-muscle invasive bladder cancer. Surprisingly, p53-mutated bladder cancers are also reported to respond better to adjuvant chemotherapy such as cisplatin (28). In 2011 Stadler *et al.* tried to confirm these findings with a very controversial phase III study (29). Unfortunately, the poor design of the study, due to the indiscriminate recruitment of patients, led to negative results, raising several concerns in the scientific community (30-32) and leaving us without a firm conclusion. "Satellite" genes of p53 belonging to the p53 pathway are also susceptible to potential alterations in UC. p21 for example is one of the downstream proteins whose transcription is regulated by p53 and it has a crucial role in blocking cell cycle through interaction with CDK proteins. Aberrant expression of p53 decreases the production of p21, affecting the inhibition of cell cycle and creating a great advantage for tumor growth. The regulation of p53 is another important process highly susceptible to alterations in UC. Amplification of MDM2, an inhibitor of p53, as well as homozygous deletion of its negative regulator p14, have been reported in several cases of bladder cancer (33,34).

Another important pathway that regulates cell cycle and is often reported altered in UC is the Rb pathway. The retinoblastoma protein (Rb) is another well-known tumor suppressor gene and a main regulator of G1-S transition. pRb binds and inhibits several transcription factors of the

E2F family, preventing initiation of the S phase (35). pRb has also been shown to regulate many chromatin remodeling proteins like the inhibition of histone deacetylase (HDAC) protein, and reducing the transcription of proteins promoting the S phase. Several members of the Rb pathway have been reported altered in bladder cancer. Table 1.1 summarizes their role and prognostic factors in UC.

1.1.2 Effects of major alteration in bladder cancer and clinical significance

Marker/ expression in urothelial carcinoma	Normal function	Molecular pathway(s) involved	Prognostic impact
Cell-cycle regulation			
p53[a]	Inhibits G_1-S progression	p53	Increased recurrence; decreased survival; amenable to cisplatin chemotherapy
p21[b]	Cyclin-dependent kinase inhibitor	p53	Increased recurrence; decreased survival
Mdm2[c]	Mediates the proteasomal degradation of p53	p53	Increased with tumor stage and grade
p14[b]	Inhibits *MDM2*	p53	Decreased survival
p16[b]	Cyclin-dependent kinase inhibitor	Rb	Increased recurrence; decreased survival
Rb[d]	Sequesters E2F; inhibits cell-cycle progression	Rb	Increased recurrence; decreased survival
CDK4[c]	Complexes with cyclin D1; involved in the G_1-S transition	Rb	Increased with tumor stage and grade
p27[b]	Cyclin-dependent kinase inhibitor	Rb	Decreased survival
Cell death			
Fas[b]	Activation signals formation of death-inducing signaling complex; promotes apoptosis	Extrinsic apoptotic	Decreased cause-specific survival
Bcl-2[c]	Inhibits caspase activation	Intrinsic apoptotic	Decreased survival; poor prognosis with adjuvant therapy
Bax[b]	Releases cytochrome c from mitochondria; promotes apoptosis	Intrinsic apoptotic	Poor prognosis; decreased overall survival
Caspase-3[b]	Promotes apoptosis	Common apoptosis effector	Increased recurrence
Cell growth			
FGFR3[e]	Receptor for fibroblast growth factor; transmits growth signals	Ras-MAPK	Increased recurrence
EGFR[c]	Receptor for epidermal growth factor; transmits growth signals	Ras-MAPK, PI3K-Akt	Increased progression; decreased survival
ErbB-2[c]	Receptor for epidermal growth factor; transmits growth signals	Ras-MAPK, PI3K-Akt	Decreased survival
VEGFR2[c]	Receptor for vascular endothelial growth factor; transmits angiogenic signals	Ras-MAPK, PI3K-Akt	Increased with disease stage, invasion, nodal metastasis
Signal transduction			
HRAS[c]	Activates Raf and PI3K	Ras-MAPK	Increased in nonprogressing Ta tumors
PKC[f]	Activates Raf, c-Fos, NF-κB; inhibits Bad	PLC/PKC	Increased recurrence
PTEN[b]	Dephosphorylates PIP_3; antagonizes PI3K signaling	PI3K-Akt	Decreased with tumor stage and grade
Gene regulation			
STAT3[c]	Regulates gene expression; increases Bcl-2, Bcl-X_L expression	JAK-STAT	Increased recurrence; decreased survival
NF-κB[g]	Regulates gene expression	NF-κB	Increased recurrence with homozygous insertion
c-Fos[c]	Regulates gene expression	MAPK	Increased with tumor grade
c-Jun[c]	Regulates gene expression	MAPK	Increased recurrence; decreased survival

(*Continued*)

Marker/ expression in urothelial carcinoma	Normal function	Molecular pathway(s) involved	Prognostic impact
Tumor angiogenesis			
HIF[c]	Transcribes genes responsible for angiogenesis		Increased recurrence; decreased survival
VEGF[c]	Promotes angiogenesis through nitric oxide synthase	Ras-MAPK, PI3K-Akt	Increased recurrence and progression; decreased survival
TP[c]	Promotes VEGF and interleukin-8 secretion; induces MMP		Increased recurrence
uPA[c]	Degrades extracellular matrix		Increased progression; decreased survival
bFGF[c]	Growth factor stimulating angiogenesis	Ras-MAPK	Increased risk of local recurrence
aFGF[c]	Growth factor stimulating angiogenesis	Ras-MAPK	Increased with increasing stage
SF[c]	Growth factor stimulating angiogenesis		Increased compared to normal controls
TSP-1[b]	Inhibits angiogenesis	p53	Increased recurrence; decreased survival
Invasion			
E-cadherin[b]	Mediates intercellular adhesion	Cadherin	Increased recurrence and progression; decreased survival
β-catenin[b]	Links cadherins to the actin cytoskeleton	Wnt/β-catenin	Increased progression; decreased survival
α6β4 integrin[h]	Links collagen VII to the actin cytoskeleton; transduces regulatory signals	Cytoskeletal signaling	Decreased survival
MMP-2[c]	Degrades extracellular matrix		Increased recurrence; decreased survival
MMP-9[c]	Degrades extracellular matrix		Increased with tumor stage and grade
TIMP-2[i]	Antagonizes MMP function		Increased recurrence; decreased survival (?)

Table1.1: Marker/expression in urothelial carcinoma.
Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; FGFR3, fibroblast growth factor receptor 3; HIF, hypoxia-inducible factor; HRAS, protein of the Harvey rat sarcoma viral oncogene homolog gene; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor–kappa B; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol trisphosphate; PKC, protein kinase C; PLC, phospholipase C; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Rb, retinoblastoma protein; SF, scatter factor; STAT, signal transducer and activator of transcription; TIMP-2, tissue inhibitor of metalloproteinase 2; TP, thymidine phosphorylase; TSP-1, thrombospondin-1; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2. aAltered. bUnderexpressed/lost. cOverexpressed. dLost/hyperphosphorylated. eOveractivated. fOverexpressed in membrane. gPolymorphic insertion/deletion in promoter region. hLost/overexpressed. iUncertain. Adapted from ""Mitra, A. P., and Cote, R. J. (2009) Molecular pathogenesis and diagnostics of bladder cancer. *Annual review of pathology* **4**, 251-285"*"* (5) with the permission of the journal.

The ability to evade apoptosis is another crucial feature that cancer cells need to acquire to be able to progress and survive. Apoptosis can occur through 2 different pathways: the extrinsic pathway, activated by cell-death receptors on the cell surface, and the intrinsic pathway, mediated by mitochondria. In the extrinsic pathway, some aggressive types of bladder cancer have been reported to be refractory to Fas ligand-induced cell death. This observation has been

corroborated by the presence of high levels of Fas ligand in the urine of several patients and has been associated with poor prognosis and decreased survival (36,37). In the intrinsic pathway, Bcl-2 family members have been reported altered in several types of UC. Bcl-2 in particular is a protein localized on the mitochondrial membrane that inhibits the activation of the pro-apoptotic caspase cascade. The overexpression of Bcl-2 correlates with poor prognosis, advanced disease and resistance to therapies (38). Bax is another important protein that acts as an effector of apoptosis in the presence of cellular stress, due to its role in the opening of mitochondrial megapores. Alterations in Bax expression have also been associated with poor prognosis and survival (39). The most common molecular defect in apoptosis is the overexpression of survivin. First discovered in 1997 (40), survivin is controversially thought to have a cytoprotective role, both at a transcriptional and a translational level, suppressing apoptosis and promoting cell and tumor growth as well as increasing drug resistance (41). An impressive number of interactions between survivin and major signaling pathways has been reported in literature. Tight control of proliferation is due to the correlation of survivin with PI3K/AKT/mTOR pathway, STAT3, Ras, HIF-1α, HSP90 and MAPK pathway. Not surprisingly, survivin has been reported as overexpressed in a high number of cancers and is currently being investigated as a potential target for future therapies (42,43).

Growth factor receptors are also important components that regulate cell proliferation, metabolism and survival. Among these, FGFR, VEGFR, EGFR, ERBB2 and ERBB3 are probably the ones that have been investigated the most. The most relevant member of the fibroblast growth factor receptors, or FGFRs, for bladder cancer is FGFR3, whose activating mutation in UC has been linked to low grade and highly recurrent tumors (Ta-T1 stage) and is present in about 70% of those cases (12-14). One of the main functions of FGFR3 is to regulate

the Ras-MAPK pathway. Activating mutations of FGFR3 and RAS account for ~82% of all the low-grade papillary bladder cancer cases, but interestingly, those mutations seem to be mutually exclusive (44).

Epidermal Growth Factor receptors or EGFRs have also been reported altered in several bladder cancer cases. Their mutation status though, conversely to FGFR3, does not seem to correlate with tumor stage. Overexpression of this receptor has been shown to lead to a decrease in the overall survival and cancer progression (45,46). ERBB2 and ERBB3 genes encode for the human epithelial growth factor receptors (HER2 and HER3). HER2 and HER3 are activated by heterodimerization after ligand-based interaction. HER2 has been reported overexpressed in several type of cancers like breast cancer, prostate cancer and colon cancer and often is associated with poor prognosis(47). In bladder cancer ERBB2 and 3 have been reported to be either amplified or upregulated in 34% of the cases (48). Generally, HER2 alterations were reported to be lower in metastasis than in primary tumor, while no differences were observed for HER3(49). However, a recent publication observed a high frequency of ERBB2 mutations in the extracellular domain of micropapillary Urothelial Carcinoma(50).

Finally, the Vascular Endothelial Growth Factor receptor, or VEGFR, is another important receptor tyrosine kinase promoting vasculogenesis and angiogenesis. Overexpression of VEGFR2 in particular has been shown to be correlated with disease progression and muscle invasion (51). This receptor has been extensively investigated as potential therapeutic target. Combination of anti-VEGFR monoclonal antibody and chemotherapeutic agent Paclitaxel in particular, has been shown to significantly reduce metastasis formation and increase the levels of apoptosis in pre-clinical models, inducing tumor regression (52). Another targeted approach was also attempted by generating a fusion protein, combining VEGF121 with the plant toxin gelonin

(rGel) in an effort to treat bladder cancer in xenograft models. This study generated promising results, showing 60% reduction in tumor growth (53).

Upon activation of tyrosine kinase receptors, the signal is transduced by several downstream pathways that include the Ras-MAPK pathway, the PI3K pathway (that will be discussed in greater depth later in this introduction), the phospholipase C (PLC) - protein kinase C (PKC) signaling cascade, the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway and the nuclear factor–kappa B (NF-κB) pathway. PKC is associated with proliferation, differentiation and apoptosis by regulating c-Fos, NFkB, Raf and by inhibiting Bad (54). PKC overexpression has been associated with high grade UC and an increased recurrence rate in low-grade patients. Similar to PKC, the Jak-STAT pathway has also been reported altered in UC and associated with a higher rate of recurrence as well as decreased overall survival (55). NF-kB is another very important transcription factor that regulates a huge number of cellular processes such as inflammation, autoimmune response, proliferation and apoptosis (56). Nf-kB is a member of the "rapid-acting" primary transcription factors, so called because they are present in the cells in an inactive state and do not require new protein synthesis to become activated. Because of this property, the Nf-kB pathway is among the first responders to harmful stimuli like reactive oxygen species, bacterial infections or cytokines exposure. It has been reported that patients affected by superficial bladder cancer that show insertion/deletion polymorphisms in the NFKB1 promoter, have a higher risk of recurrence (57).

Many other genes involved in tumor angiogenesis and invasion such as HIF-1 and 2, MMPs, ß-Catenin and E-cadherin have also been reported altered in UC and have been correlated with different clinical outcomes. A description of these proteins is shown in table 1.1 and a detailed review is available on-line (5).

1.1.3 The Cancer Genome Atlas study and bladder cancer

In March 2014 Nature published a very important study on bladder cancer, as part of a bigger project named "The Cancer Genome Atlas" or TCGA. In this work the authors characterized 131 high-grade muscle invasive tumors (T3-T4) from chemotherapy naïve patients, taking into account DNA copy number, somatic mutations, messenger RNA and microRNA (miRNA) expression, protein and phosphorylated protein expression, DNA methylation, transcript splice variation, gene fusion, viral integration, pathway perturbation, clinical correlations and histopathology (48). The samples displayed a large number of DNA alterations, scoring bladder cancer as the type of cancer with the third highest number of mutations in the TCGA study after lung cancer and melanoma (Figure 1.5a). The study was able to confirm many of the observations previously reported in literature about bladder cancer. On top of that, they reported novel statistically significant altered genes. Novel mutations reported in Figure 1.5b include MLL2 (also called KMT2D; 27%), CDKN1A* (14%), ERCC2* (12%), STAG2 (11%), RXRA* (9%), ELF3* (8%), NFE2L2 (8%), KLF5* (8%), TXNIP (7%), FOXQ1* (5%), RHOB* (5%), FOXA1 (5%), PAIP1* (5%), BTG2* (5%), ZFP36L1 (5%), RHOA (4%) and CCND3 (4%). Nine of those (the ones marked with an asterisk) were unique for bladder cancer and not identified in other TCGA subsets. The study also reported novel amplifications and deletions, like those in the genes PVRL4, BCL2L1 and ZNF703 (Figure 1.5c). From the RNA seq-analysis of 129 tumors, the authors were able to identify 4 different clusters (Figure 1.6). Cluster I identified as "papillary-like", showed high levels of FGFR3 mutations and alterations compared with the other 3 clusters, similar to what was observed in low-grade papillary bladder cancer tumors (Figure 1.6a). Both Cluster I and II were shown to be enriched in estrogen receptors

ERBB2 and ESR2, both at the protein and mRNA level, similar to HER2-positive breast cancer and luminal breast cancer (Figure1.6d).

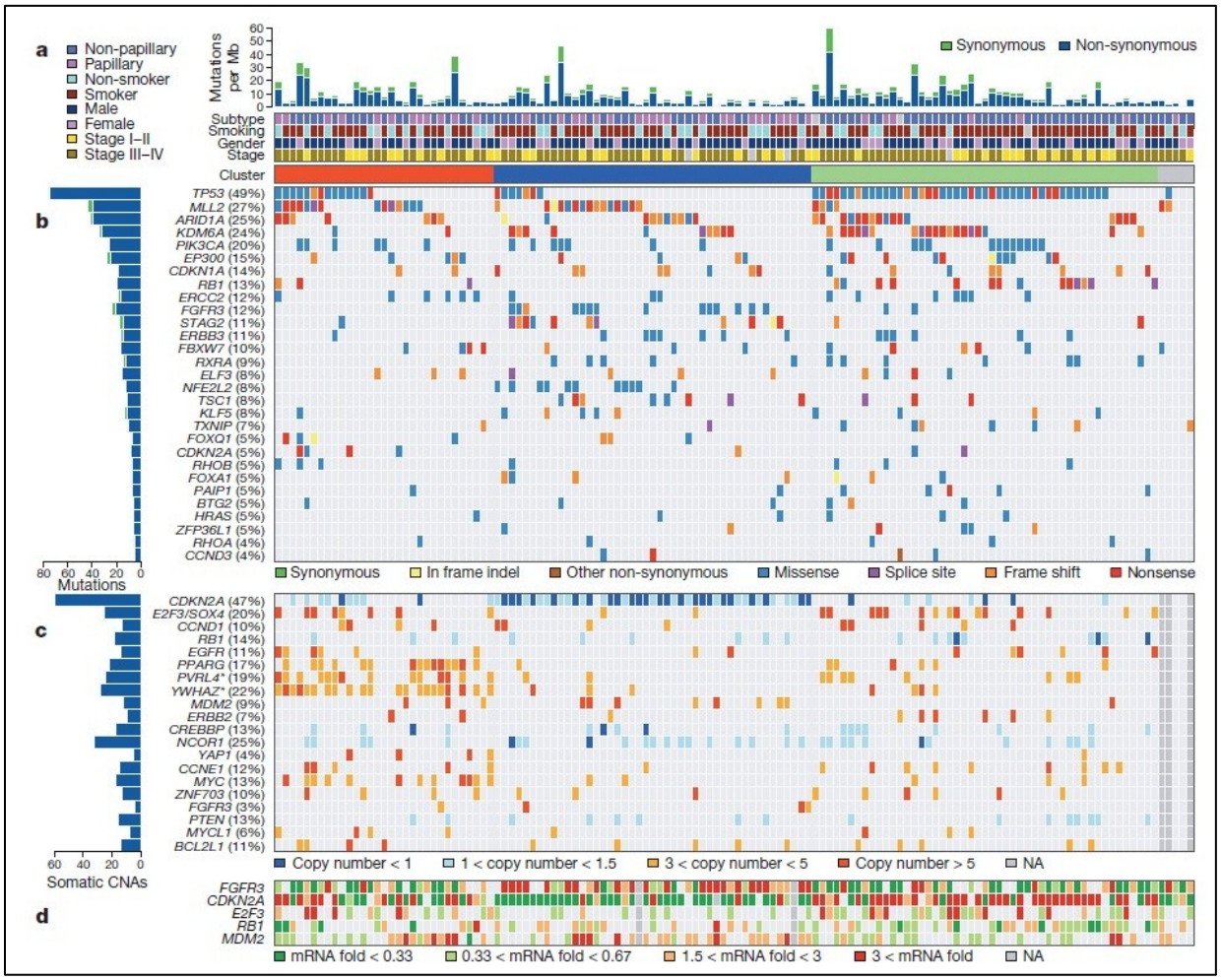


Figure1.5: The genomic landscape of bladder cancer.
a, Mutation rate and type, histological subtype, smoking status, gender, tumor stage and cluster type. b, Genes with statistically significant levels of mutation (MutSig, false discovery rate ,0.1) and mutation types. c, Deletions and amplifications for genomic regions with statistically significant focal copy number changes (GISTIC2.0). 'Copy number' refers to absolute copy number. Note that two amplification peaks (*) contain several genes, any of which could be the target, as opposed to the single gene listed here. d, RNA expression level for selected genes, expressed as fold change from the median value for all samples. Tumor samples were grouped into three clusters (red, blue and green) using consensus NMF clustering (see the main text and Supplementary Fig. 2.1.2). Three samples with no copy number data and two samples with no mutations in the genes were not used in the clustering and are shown in grey. Adapted from "Cancer Genome Atlas Research, N. (2014) Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**, 315-322" (48) with the permission of the journal

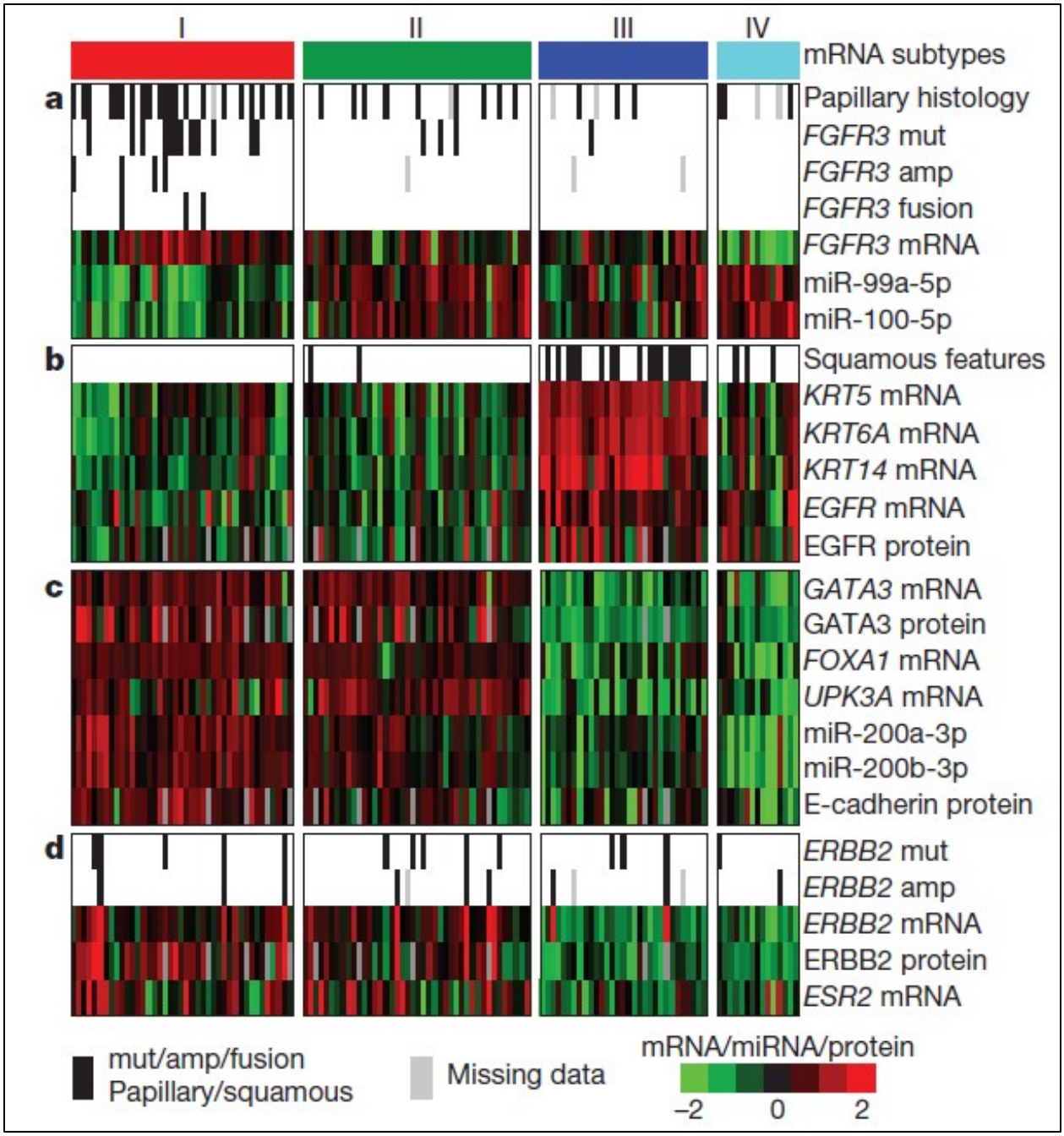


Figure1.6: Expression characteristics of bladder cancer.
Integrated analysis of mRNA, miRNA and protein data led to identification of distinct subsets of urothelial carcinoma. Data formRNA, miRNAand protein were z-normalized, and samples were organized in the horizontal direction by mRNA clustering. a, Papillary histology, FGFR3 alterations, FGFR3 expression and reduced FGFR3-related miRNA expression are enriched in cluster I. b, Expression of epithelial lineage genes and stem/progenitor cytokeratins are generally high in cluster III, some of which show variant squamous histology. c, Luminal breast and urothelial differentiation factors are enriched in clusters I and II. d, ERBB2 mutation and oestrogen receptor beta (ESR2) expression are enriched in clusters I and II. Adapted from "Cancer Genome Atlas Research, N. (2014) Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**, 315-322" (48) with the permission of the journal

Cluster III was labeled as “basal/squamous like” because of its enrichment in basal biomarkers and squamous histopathological features similar to what was observed in basal-like breast cancer, squamous cell head and neck cancer and lung cancer. They concluded their analysis stating that all these observations suggested how bladder, breast, lung and head and neck cancer may share the same development pathway despite their different origins.

Our lab recently published a detailed work partially related to the TCGA study (58). We showed how within a cohort of 73 patient samples that developed muscle-invasive bladder cancer, we distinguished 3 specific subsets very much alike to what was shown in the TCGA study (Figure 1.7). Our luminal subset is very close to what was reported as subset I (papillary-like) in the TCGA study, and shares the same common features: FGFR3 alteration, E-cadherin and miR200 family expression and were characterized by PPARγ and estrogen receptor expression. The p53-like subset is very similar to subset II of the TCGA study and features luminal markers, but with a wild-type p53 gene expression signature. Finally, our basal subset overlaps with subset III and IV of the TCGA study, displaying squamous features and overexpression of KRT5, KR6, KRT14 and CDH3 and was characterized by activated p63, an homolog protein of p53, well known for being implicated in the development of epithelial tumors (59). In addition to what was published by the TCGA authors, we linked our 3 different subsets with clinical significance, showing how patients having basal type tumors presented with a more aggressive disease and significantly lower survival compared to luminal and p53-like tumors. Another important observation reported in that paper was the significant resistance of the p53-like tumors to conventional chemotherapy, observed in patients and confirmed in cancer cell lines.

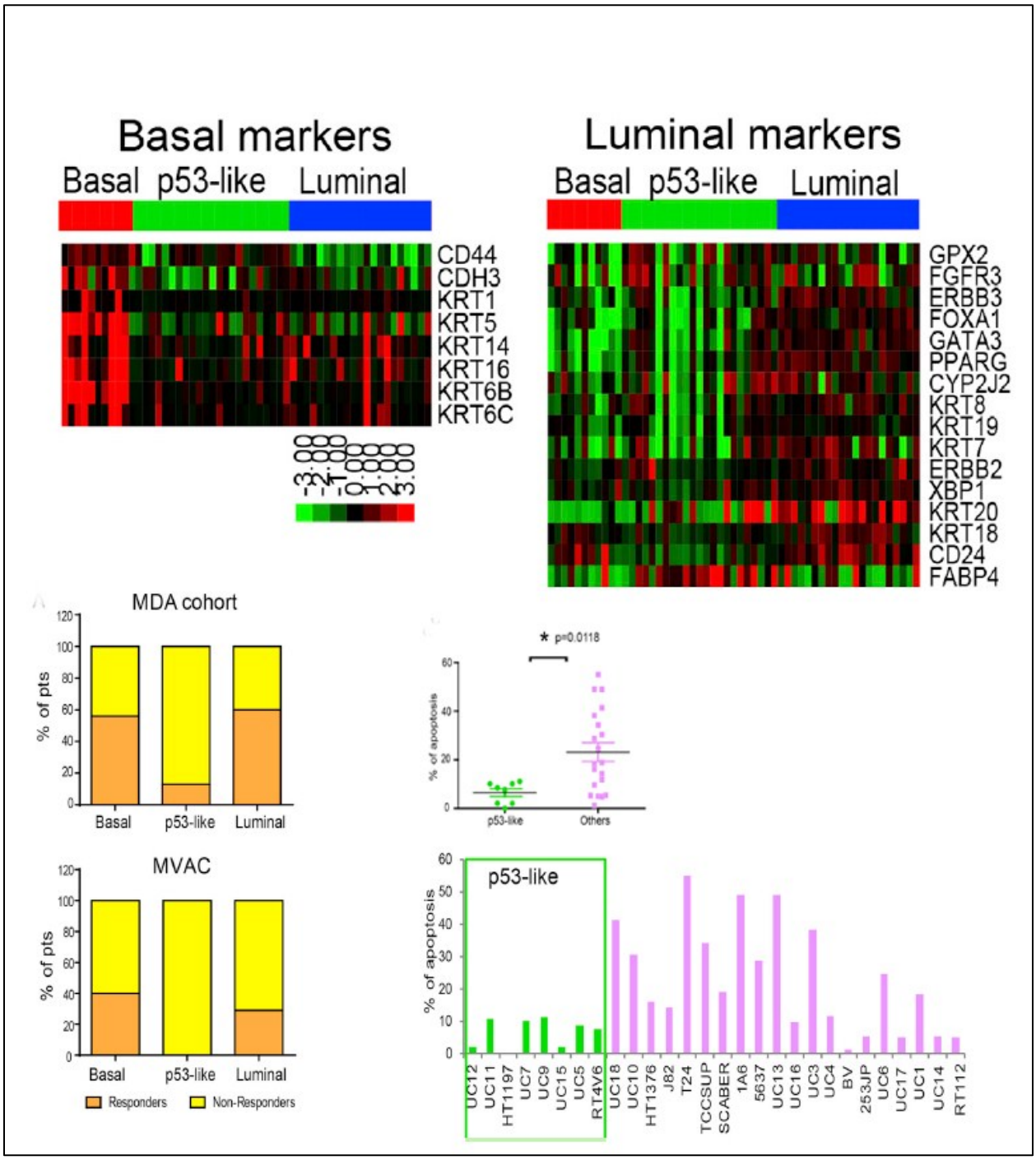


Figure1.7: Subset of muscle invasive bladder cancer published by Choi *et al.* discriminating between 3 different subsets based on gene expression profiling. P53-like subset is shown to be more resistant to conventional chemotherapy in both patients and cell lines. Adapted from "Cancer Genome Atlas Research, N. (2014) Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**, 315-322" (48) with the permission of the journal.

In light of what was discussed so far, dividing bladder cancer into just two big subgroups: muscle invasive and non-muscle invasive, is very reductive. As pointed out by Carolyn D. Hurst and Margaret A. Knowles in their recent Cancer Cell preview (60), it would perhaps be more appropriate to let the genetic signature of the tumors guide us towards more specific prognosis, patient stratification and treatment. In February 2014, Damrauer *et al.* published a PNAS paper in which they distinguished between two major subtypes within high grade MIBCs: "luminal" and "basal-like" subtypes (61). They reported a list of 47 predictor genes (BASE47) that they used to classify the 262 muscle invasive tumors involved in their study. They effectively showed how BASE47 provided a good representation of tumor biology, as well as correlated with prognostic values. In this study, Damrauer *et al.* also remarked on genetic similarities between basal-like bladder cancer and basal-like breast cancer by showing how similar genetic subtypes were closely deregulated in these two types of cancer.

In a manuscript recently submitted to Nature, our colleague Woonyoung Choi made a comparison between our study, the TCGA study and Damrauer's paper. In this manuscript, we showed how our basal subset, overlaps almost perfectly with Damrauer's basal subset and with the TCGA's cluster III. The p53-like subset that we described in our Cancer Cell publication, as mentioned above, overlaps almost perfectly with TCGA's cluster II, while the study from the University of North Carolina did not take into account any p53-like feature. Because of this reason, tumors from both their luminal and basal subset fell in our p53-like group. Finally, we showed how our luminal subset almost perfectly overlaps with Damrauer's luminal subset and TCGA's cluster I. All of these observations corroborate the idea that Carolyn D. Hurst and Margaret A. Knowles expressed in their Cancer Cell preview article; they claimed that the definition of "muscle invasive bladder cancer" is indeed an obsolete way to describe multiple

types of bladder cancer. This definition in fact, encompasses at least 3 different types of disease, which can be identified by distinct biomarkers and, lead to different prognosis, and should be approached and treated in different and more specific ways.

1.2 The PI3K pathway

1.2.1 Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway (Figure 1.8) is an important cellular pathway activated by several receptor tyrosine kinases that regulates many important biological processes like metabolism, protein translation, proliferation and survival (62). PI3K is composed of two subunits p85 and p110-alpha. p85 has mainly regulatory properties while p110 acts as a catalytic subunit. Upon activation, p85 induces p110 to phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). This specific process is reversed by the phosphatase, PTEN, that acts as a suppressor of the PI3K pathway. PIP3 then phosphorylates AKT on its T308 residue through the activation of protein dependent kinase-1 (PDK1). AKT or protein kinase B (PKB) is another very important element of the PI3K pathway as it directly regulates pro-survival stimuli through the activation of Bad, CREB, Forkhead family, IKK and Mdm2 and the inhibition of Procaspase 9 and Ask1. AKT can also be activated by phosphorylation on residue Ser473 by mTORC2 and DNAPK in the nucleus (63,64). AKT also regulates cell cycle progression and cell growth by directly modulating GSK3, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ and by inhibiting TCS2 that in turn inhibits RHEB, a positive regulator of mTORC1 (65). mTORC1 itself mediates several more cell processes like cell cycle progression, proliferation angiogenesis and autophagy (66). The PI3K pathway plays a crucial role in cellular metabolism in the presence of abundant nutrients and it is easy to understand

what a crucial role this pathway may play in cancer development. In many types of malignancies the PI3K/AKT/mTOR pathway has in fact been reported to be altered, including bladder cancer.

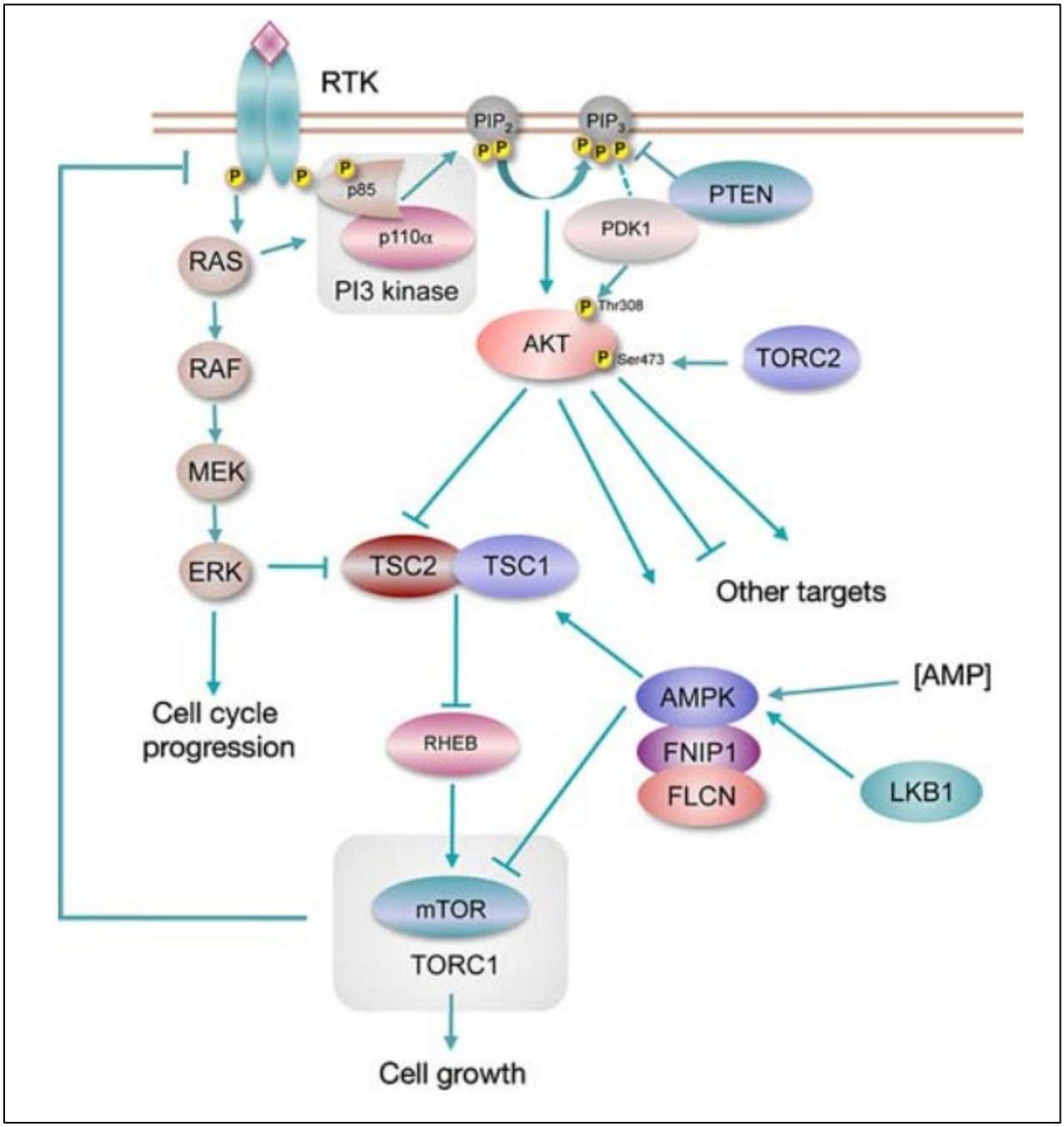


Figure 1.8: The class IA PI3K signaling pathway. Adapted from "Knowles, M. A., Platt, F. M., Ross, R. L., and Hurst, C. D. (2009) Phosphatidylinositol 3-kinase (PI3K) pathway activation in bladder cancer. *Cancer Metastasis Rev* **28**, 305-316" (11) with the permission of the journal.

1.2.2 PI3K pathway and bladder cancer

The PI3K/AKT/mTOR pathway has been reported altered in bladder cancer as well as in many other types of cancer (67,68). Among its key regulators, the tumor suppressors PTEN and LKB1 have been found to be often deleted or mutated with consequential reduction of their negative regulatory activity (69,70). AKT1, AKT2, PDK1 and PIK3CA, on the other hand, have been found to be upregulated or constitutively activated (71-74). The data shown in Figure 1.9, derived from the recently published TCGA study (48), highlight how 107 of 125 bladder cancer patients (~86%) had alterations in one or more proteins belonging to the PI3K pathway; the most represented of which was PI3CA, being altered in ~32% of the tumors.

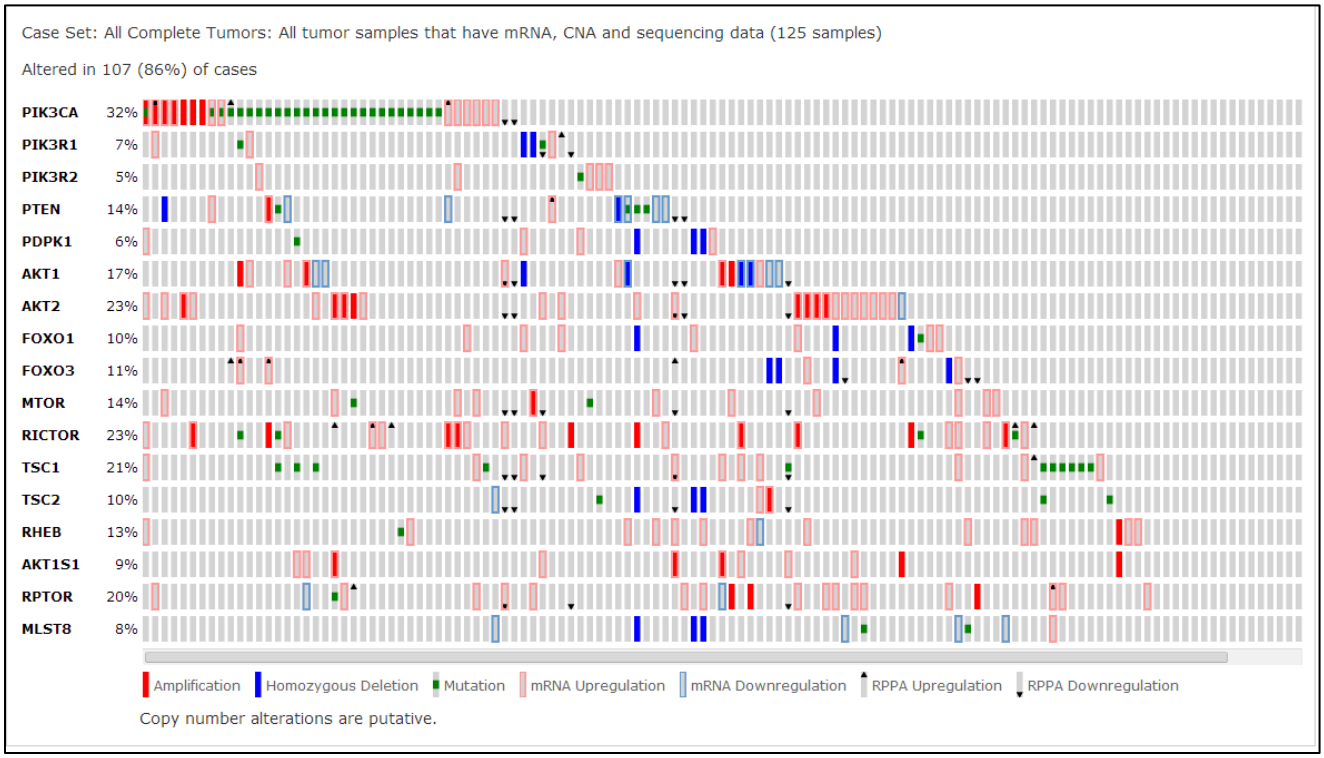


Figure1.9: Representation of all the alterations reported in the PI3K pathway by the TCGA study (48) generated by using the software provided at this url: http://www.cbioportal.org/public-portal/

PIK3CA, whose structure is shown in figure 1.3a, has been found to be mutated or activated in numerous types of tumors (75). In bladder cancer PIK3CA mutations seem to be associated with low tumor grade (76). Surprisingly, the distribution of mutations for this protein is significantly different when comparing bladder cancer (77) to other types of tumors (Figure 1.10) in the COSMIC database (www.sanger.ac.uk/genetics/CGP/cosmic/), suggesting that the spectrum of mutations for the PI3K pathway in cancer is tissue-specific rather than conserved.

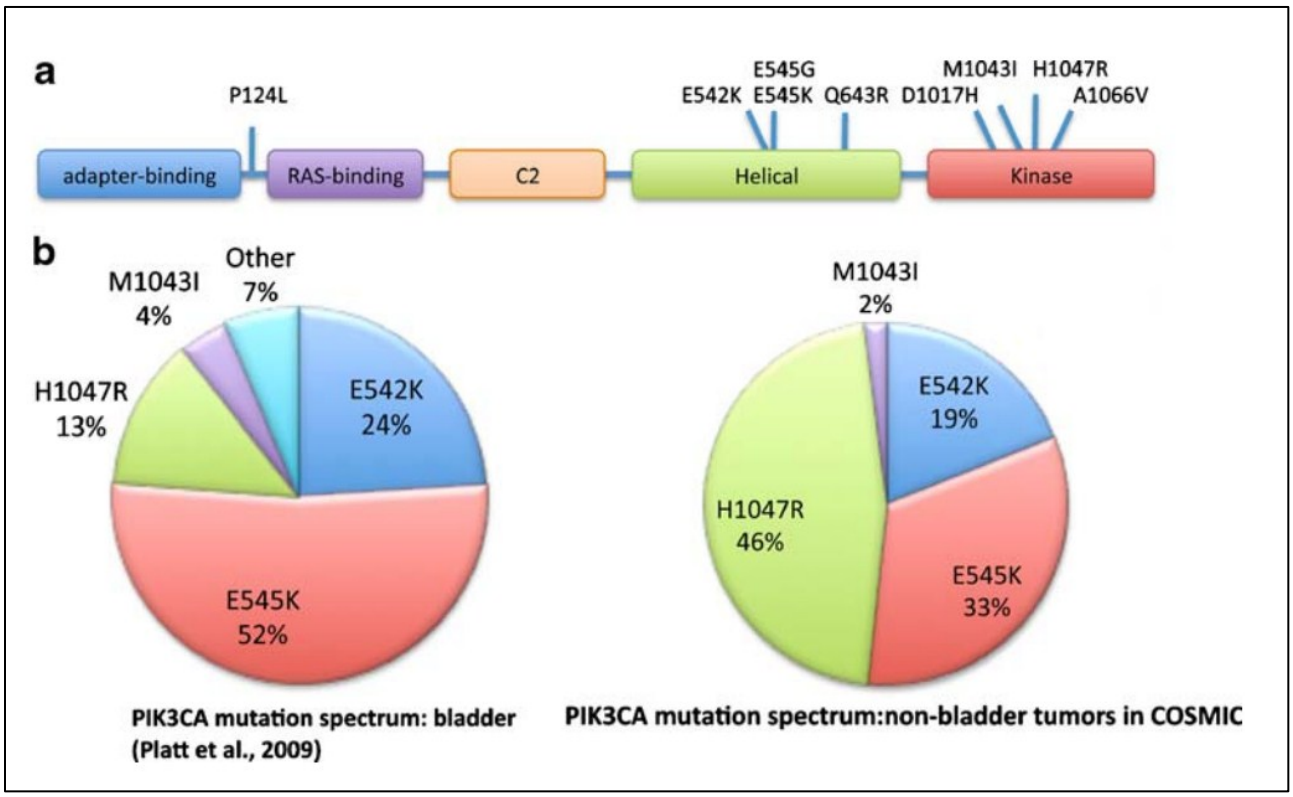


Figure 1.10: Mutations of PIK3CA identified in bladder cancer.
A schematic representation of the protein showing functional domains and the position of mutations identified to date. Both the adapter-binding and C2 domains interact with p85. B Comparison of PIK3CA mutations in bladder cancer and those listed in COSMIC for positions M1043, H1047, E542 and E545, excluding bladder tumor mutations. Adapted from "Knowles, M. A., Platt, F. M., Ross, R. L., and Hurst, C. D. (2009) Phosphatidylinositol 3-kinase (PI3K) pathway activation in bladder cancer. *Cancer Metastasis Rev* **28**, 305-316" (11) with the permission of the journal.

1.3 The Insulin-like growth factor receptor signaling pathway

1.3.1 Introduction and overall structure of the receptor

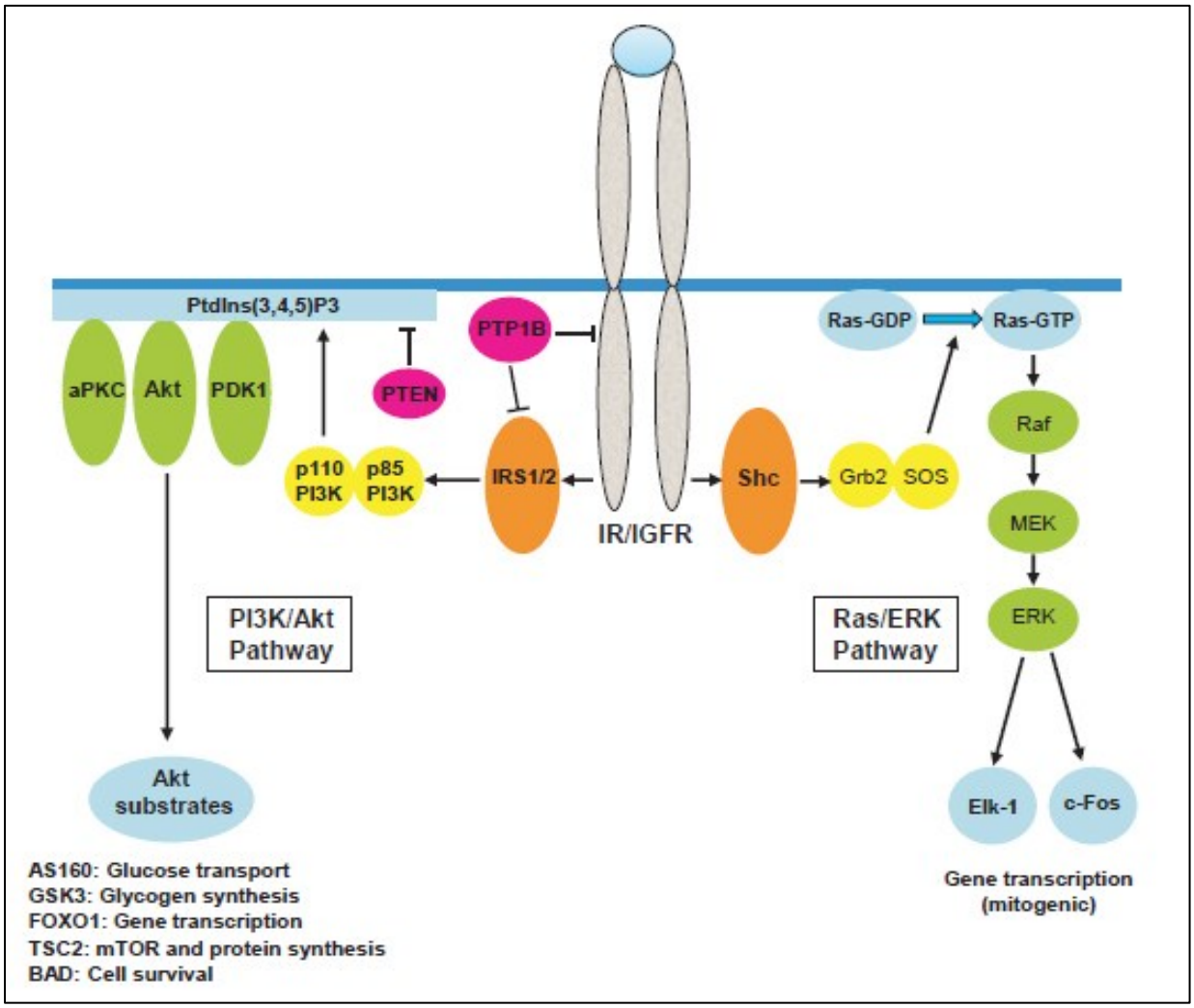


Figure 1.11: Canonical insulin/IGF–1 signaling pathways.
The main components of the PI3K/Akt and Ras/ERK pathways, including receptors, substrates (in orange), adaptors and transducers (in yellow), serine/threonine kinases (in green), downstream components (in blue) and some negative regulators (in purple) are shown. Published from "Desbuquois, B., Carre, N., and Burnol, A. F. (2013) Regulation of insulin and type 1 insulin-like growth factor signaling and action by the Grb10/14 and SH2B1/B2 adaptor proteins. *The FEBS journal* **280**, 794-816" (81) with the permission of the journal.

Insulin and insulin-like growth factors regulate many physiological processes inside the cell but play distinct roles: while insulin mainly regulates the metabolism of glucose and lipids in muscle, fat and the liver, regulating the uptake and storage of these bioproducts, insulin-like

growth factors or IGFs mainly promote cell growth and differentiation (78,79). IGF-2 is mainly found during fetus development, while IGF-1 is broadly expressed after birth and throughout our entire life (80).

Insulin receptors (IRs), as well as insulin-like growth factor receptors (IGF-1Rs) are both similar to the class II RTK family (82) and are both composed of 2 alpha and 2 beta subunits. The alpha subunits are responsible for binding the ligand extracellularly, while the beta subunits contain the tyrosine kinase domains that activate the receptor's downstream pathways. Both subunits are synthetized from the same mRNA precursor and undergo posttranslational modification by proteolytic cleavage and disulfide linkage to form the final functionally active subunits (83). The extracellular domains of the IRs and IGFRs are very similar in structure, but characterized by mild differences that give each receptor its specificity (84). Despite the fact that those receptors are dimers, one ligand can bind each single receptor (85). The intracellular domains are constituted by a tyrosine kinase domain and a juxtamembrane domain about 250 and 45 amino acids long, respectively, and having 84 and 65 percent homology between IR and IGFR (82). Trans-autophosphorylation in the tyrosine kinase domain activates the receptor as well as facilitates the binding of the adaptor protein Grb10/14 and PAS/SH2 (86-90). Phosphorylation in the juxtamembrane portion of the receptor becomes the binding site for other adaptor proteins like IRSs and Shc (91).

1.3.2 Downstream pathways and signaling modulation

Upon binding with its ligands, the IR and IGFR activate two main pathways: the PI3K/AKT pathway and the MAPK pathway (Figure 1.11) (92-94). The PI3K pathway discussed above, mainly regulates metabolism, but also survival and proliferation (95), while the MAPK pathway

has little or no effect on metabolism, but tightly regulates proliferation and differentiation (96,97). IRs/IGF-1Rs are regulated by a plethora of different substrates and adaptor proteins with both positive and inhibitory potential. Perhaps the most known class of substrate proteins is the IRS family. IRS-1 and 2 are broadly expressed in mammals, while IRS-3 and 4 are more tissue specific. Once activated, IRSs interact directly with PI3K through their Src homology 2 domain (SH2) (98). Interestingly, the role of IRS-1 seems to be different depending on the type of receptor this substrate is coupled with. When activated by IR, IRS-1 seems to preferably regulate PI3K while, when coupled with IGFR, it seems to regulate Grb2 (99). The differential engagement and regulation of IR and IGFR substrates and adaptor proteins is still not completely elucidated. Grb2-associated binders (Gabs) and downstream of kinases (DOKs) for example seem to act as alternatives to IRSs in cells that do not express high levels of IRSs and are highly tissue and pathway specific (100,101). The peculiar behavior of these proteins in different types of cells could very well be the potential explanation for what is described in Chapter 5 of this thesis. Shc is another very important adaptor protein for the signal transduction of IRs and IGFRs. ShcA is present in 3 different isoforms (p66, p52 and p46) derived by alternative splicing of the same precursor and has MAPK pathway activating properties in the case of p52/p46 and inhibitory roles in the case of p66 (102-104). Once activated, Shc can recruit Grb2 and SOS, promoting MAPK pathway activation. Whether the effects of insulin or IGF binding to their respective receptors will determine an increase in cell metabolism or in mitosis rate is determined by the competition between IRS and Shc. Higher expression of the former translates into activation of PI3K; vise-versa, higher expression of the latter will increase mitogenic stimuli by MAPK pathway activation (105).

1.4 Autophagy

1.4.1 Introduction. Autophagy is a Greek derived word that literally means "self-eating" and underlies a catabolic cellular mechanism constantly active in any cell at basal levels. However, it is dramatically activated upon cell starvation and nutrient deprivation (106-110). During autophagy, a number of sub cellular components, harmful bio-products, as well as invading micro-organisms, such as viruses and bacteria, are targeted and degraded (111-114). While in the past the role of autophagy has been described as an alternative cell death mechanism driven by the accumulation of autophagosomes (115), modern studies still have not fully elucidated the whole process (116,117). More apparent is the survival benefit that this process provides to the cells in response to starvation, environmental stress (e.g. oxidative stress) or protein aggregate accumulation during the maintenance of regular cell homeostasis (106). During autophagy, designated targets, such as surplus molecules, damaged organelles, misfolded proteins, harmful bioproducts, etc. are assimilated into a double layer vacuole called an autophagosome that will subsequently merge with lysosomes. The undesired cargo will then be completely degraded by lysosomal proteases (108,118,119). The molecular cascade triggering autophagy is an intricate process that could be simplified by describing it as a four step event composed of: vesicle nucleation, vesicle elongation, docking and fusion, and degradation. Each one of these steps is tightly regulated by a plethora of different proteins that will be described in greater detail later in this chapter. As previously anticipated, mTOR is one of the major repressors of autophagy. In the presence of abundant nutrients and growth factors, the activation of the PI3K pathway by insulin-like receptors, as well as other growth factor receptors, promote cell proliferation and protein synthesis, shutting down at the same time autophagy as representative of the major cellular catabolic process (120). Other major regulators of autophagy are: the eukaryotic

initiation factor 2α (eIF2α), activated upon nutrient starvation, double-stranded RNA, and endoplasmic reticulum (ER) stress; 5'-AMP-activated protein kinase (AMPK), in response to low energy; BH3-only proteins that disrupt Bcl-2/Bcl-X_L inhibition of the Beclin 1/class III PI3K complex; the tumor suppressor protein, p53; death-associated protein kinases (DAPk); the ER-membrane-associated protein, Ire-1; the stress-activated kinase, c-Jun-N-terminal kinase; the inositol-trisphosphate (IP_3) receptor (IP_3R); GTPases; Erk1/2; ceramide; and calcium (106,121-123).

1.4.2 The steps of autophagy.

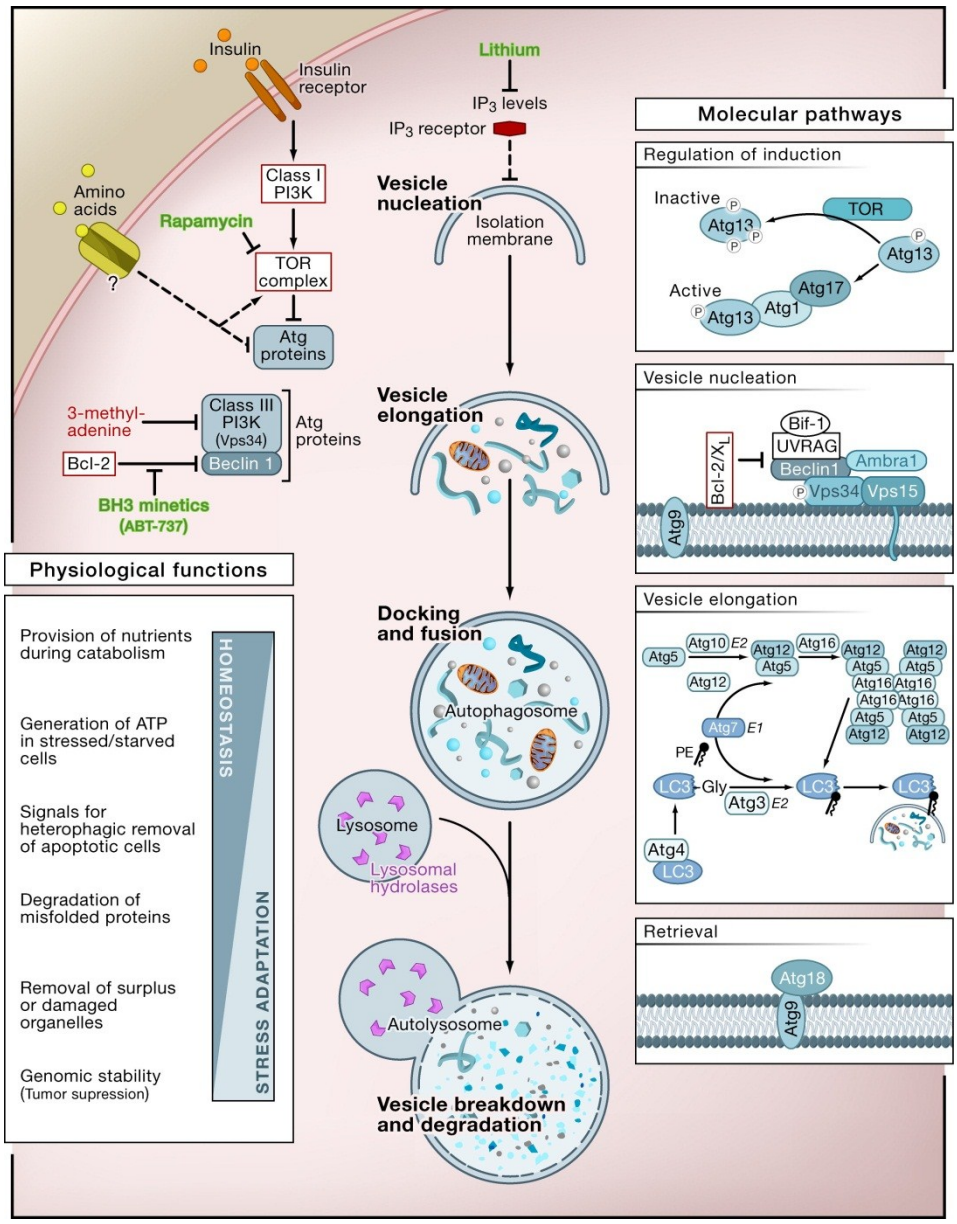


Figure1.12: The Cellular, Molecular, and Physiological Aspects of Autophagy.
The cellular events during autophagy follow distinct stages: vesicle nucleation (formation of the isolation membrane/phagophore), vesicle elongation and completion (growth and closure), fusion of the double-membraned autophagosome with the lysosome to form an autolysosome, and lysis of the autophagosome inner membrane and breakdown of its contents inside the autolysosome. This process occurs at a basal level and is regulated by numerous different signaling pathways. Shown here are only the regulatory pathways that have been targeted pharmacologically for experimental or clinical purposes. Inhibitors and activators of autophagy are shown in red and green, respectively. At the molecular level, Atg proteins form different complexes that function in distinct stages of autophagy. Shown here are the complexes that have been identified in mammalian cells, with the exception of Atg13 and Atg17 that have only been identified in yeast. The autophagy pathway has numerous proposed physiological functions.
Adapted from "Levine, B., and Kroemer, G. (2008) Autophagy in the pathogenesis of disease. *Cell* **132**, 27-42" (106) with the permission of the journal.

As anticipated, autophagy is a multistep process regulated by more than 20 genes (Figure 1.12). The regulation of induction is the first critical process and mainly happens downstream of mTOR through the interaction between Atg13, Atg1 and Atg17 when the complex is in its activated form. mTOR can directly inhibit autophagy by phosphorylating ATG13 on multiple residues and disrupting the initiating complex. The second step is nucleation, the process of autophagosome membrane formation, and is the least understood phase of autophagy. The initiation of vesicle formation does not seem to form by budding from preexisting organelles, but they rather seem to form *de-novo*, probably from the ER. The main complex responsible for vesicle nucleation is the (PtdIns) 3-kines complex I, formed by multiple proteins including Vps34, Vps15, Beclin1 Ambra1 and Atg9 (124). After the vesicle nucleation is over, the next step is vesicle elongation, a complicated process mediated by two ubiquitin-like (Ubl) proteins, ATG8 and ATG12 that are activated by ATG7, a homolog to the E1 ubiquitin activating enzyme. ATG8 undergoes two posttranslational events, resulting in binding to phosphatidylethanolamine (PE) and Atg12. This complex then gets covalently bound to Atg5 to subsequentially initiate the formation of a multiprotein complex constituted by several Atg12, Atg5 and Atg16 units. This complex will then facilitate the recruitment of microtubule-associated protein 1A/1B-light chain 3 (LC3), previously conjugated with PE by its interaction with Atg4 and ATg3, to the autophagosome membrane (125). Upon formation of mature autophagosomes, a retrieval pathway mediates the disassembly of ATG proteins for their recycling in novel autophagosome formations.

The retrieval step is operated by the interaction of Atg2 and Atg18 (126-129). At this stage the targeted molecules are docked and fused in the mature autophagosome. This process is accurately reviewed by Wang and Klionsky (130) and involves complicated molecular

machinery, including the SNARE proteins Vam3, Vam7, Vti1, and Ykt6; the NSF, SNAP, and the GDI homologs Sec17, Sec18, and Sec19, the rab protein Ypt7 and members of the class C Vps/HOPS complex. Finally, mature autophagosomes are ready to fuse with lysosomes for the final step of vesicle breakdown and degradation, a process that is tightly regulated by Atg15 (131). The acid pH of the vacuole lumen plays a crucial role for the fusion of the lysosome with the autophagosome. This process is the crucial stage where Chloroquine, the chemical inhibitor utilized in this dissertation as a tool to block autophagy activation in bladder cancer cells, prevents endosomal and lysosomal acidification, ultimately stopping the fusion between lysosomes and autophagosome (132).

1.4.3 Autophagy and cancer.

Despite the fact that autophagy was first described in the early 1960s, only recently did the scientific community really start to grasp the great importance of this process in cancer. It is easy to understand why earlier studies described autophagy as a tumor suppressor mechanism. Being downregulated by mTOR, a member of the well-known pro-carcinogen PI3K pathway, and therefore being suppressed in the situation of active proliferation and fast paced metabolism, the first impression was that inhibition of autophagy would favor cancer tumorigenesis (133). More evidence supporting this thesis came from other studies showing that transgenic mice lacking the expression of Atg genes were more prone to develop fibrosarcomas upon exposure to carcinogens and more susceptible to develop many types of tumors compared to their wild type counterparts (134-137). Moreover, heterozygous disruption of Beclin1, one of the many regulators of autophagy (see 1.3.2), was observed in ovarian, breast and prostate cancer (138) and its re-expression resulted in the suppression of tumorigenesis in breast cancer cell lines (139). Even monoallelic deletion in Beclin1 was reported to be associated with an increased risk

of liver and lung carcinomas, lymphoma and mammary hyperplasia in aging mice (133,140,141). Only in recent years, did many studies start to show how autophagy is indeed a double-edged sword for cancer development. While it is true that in certain situations, like the ones just mentioned above, the inhibition of autophagy could be beneficial for tumorigenesis, it is important to remember that autophagy is also a cytoprotective, pro-survival mechanism that plays an important role in cancer cells, that by definition are prone to starvation, hypoxia, aerobic metabolism and genomic instability, a phenomenon known as Warburg effect (142). Recent publications show how overexpression of known oncogenes, like K-Ras and the loss of tumor suppressor genes, such as p53, are tightly linked with upregulation of autophagy (143,144). Surviving hypoxic conditions is crucial for cancer development. Such mechanisms, tightly regulated by HIF-1α, produce high levels of reactive oxygen species (ROS), whose accumulation is very toxic for cells. It has been shown how HIF-1α regulates, among others, the activation of regulatory genes of autophagy like BNIP3 and suppresses mTOR by activating REDD1 (145,146). The activation of autophagy is crucial for the degradation of ROS and cell survival. On the other hand, the accumulation of ROS in autophagy deficient cancer cells leads to the accumulation of p62 accumulation, which further leads to NF-kB pathway inhibition and liver damage that in turn promotes hepatocellular carcinoma (HCC) (147). ER stress, caused by ROS and protein aggregates accumulation, can also be beneficial to cancer cells by activating DNA damage response mechanisms that would counteract the high genomic instability of cancer cells. Another tumorigenic effect of autophagy deficiency for cancer cells is that the accumulation of protein aggregates and ROS may lead to cell death driven by necrosis. This event would benefit cancer development by inducing inflammation, contributing to the formation of a beneficial microenvironment (148). The study of autophagy in relation to cancer development is a

fascinating area of study. Trying to fully understand a mechanism that can potentially benefit and harm tumorigenesis is challenging, but also rewarding at the same time. One of the main focuses of this thesis is indeed trying to use the inhibition of autophagy to our advantage, with the ultimate goal of sensitizing bladder cancer cells to target therapies.

1.5 Current targeted therapies in bladder cancer

As extensively outlined in the previous subchapters of this thesis, as of today the pharmaceutical gold standards of bladder cancer treatment are the same as the ones that were available about 30 years ago. Novel targeted therapies for the treatment of this disease are urgently needed. Many targeted therapy approaches have been proven to be effective for several types of cancer so far, including breast, lung and colon cancer (149-151). The observations described by the TCGA study as well as the ones published by Choi *et al.*, (58) highlight many potential hot spots for targeted therapy. Table 1.2 summarizes the current ongoing clinical trials that focus on small molecule inhibitors or immunotherapy.

Because of the high mutation/amplification rate of ERBB2 in muscle invasive bladder cancer (~8% in the TCGA database) and because of its success in the treatment of breast cancer, monoclonal antibody trastuzumab has been employed in a single-arm clinical trial. Despite the results , which show 70% rate of response, minor or no effect on overall survival was reported (152). Perhaps a better result could have been achieved if the study targeted those patients with tumors harboring ERBB2 mutations or amplifications. In the era of personalized medicine clinical studies should always be carefully planned to maximize the beneficial effects of targeted therapies by selecting the appropriate subset of patients. Alternative strategies to target ERBB2 were attempted by combining lapatinib, an EGFR and HER2 inhibitor with conventional chemotherapy or by directly targeting HER2 using it as a target for immunotherapy.

Ongoing clinical trials of therapies targeted at actionable mutations in muscle-invasive bladder cancer

Targeted mutation	Agent under investigation	Patient eligibility	NCT number	Trial design	
HER2	Trastuzumab	HER2 ≥2+ by IHC	NCT00238420	Chemoradiotherapy +/- trastuzumab	Phase I/II
HER2	DN24-02	HER2 ≥1+ by IHC	NCT01353222	Adjuvant DN24-02 vs observation	Phase II – randomized
EGFR	Erlotinib	No EGFR specific criteria	NCT00749892	Neoadjuvant erlotinib x 3-5 weeks	Phase II – single arm
EGFR	Erlotinib	No EGFR specific criteria	NCT00380029	Neoadjuvant erlotinib × 4 weeks then continue for 2 years postop	Phase II – single arm

EGFR, epidermal growth factor receptor; IHC, immunohistochemistry. HER2, human epidermal growth factor receptor 2.

Table1.2: Ongoing clinical trials of therapies targeted at actionable mutations in muscle-invasive bladder cancer. Adapted from "Bambury, R. M., and Rosenberg, J. E. (2013) Actionable mutations in muscle-invasive bladder cancer. *Current opinion in urology* **23**, 472-478" (153) with the permission of the journal.

The Epithelial Growth Factor Receptor is another target that is currently under investigation for targeted therapy against bladder cancer. Monoclonal antibodies (cetuximab/panitumumab) as well as a small molecule inhibitor (erlotinib/gefitinib) are currently employed in phase II clinical trials. The cetuximab study showed a 25% response rate, but no improvement in the outcome when used in combination with gemcitabine/cisplatin (154). Once again, a more specific trial aiming to a specific subset of patients with basal types of tumors could have perhaps generated better results. Unfortunately, no response was observed during the gefitinib study, despite the promising results shown on non-small cell lung cancer (155,156). Despite these negative results two more clinical trials are currently ongoing, investigating the effects of erlotinib in the neo-adjuvant setting (153).

As previously mentioned, FGFR3 mutations are extensively reported in many low-grade tumors, as well as in the luminal subset of muscle invasive bladder cancer patients. A targeted therapy focused on this receptor is therefore a really attractive strategy to treat this group of patients. Despite that, a clinical trial evaluating the effects of dovotinib, a small molecule inhibitor targeting FGFR3, showed disappointing results on both wild-type and mutated FGFR3

patients. Some scientists argued that better drugs could have provided better results in this study. Dovotinib in fact, also generated disappointing outcomes in other malignancies, where subsequent trials with improved FGFR inhibitors showed improved results. Because of these results, improved FGFR inhibitors are currently on trial for the treatment of bladder cancer.

In section 1.2.3, we reported TCGA data showing how ~86% of MIBC patients display alterations in the PI3K/AKT pathway. Unfortunately, once again, clinical trials with the mTOR inhibitor everolimus showed limited results on most of the patients treated with this drug. Noteworthy, however is the fact that a small cohort of patients did benefit from this kind of therapy strengthening the idea that personalized cancer therapy could be the answer to defeating this disease (157). Further studies showed PI3K H1047R mutations as well as the tuberous sclerosis complex 1(TSC1) inactivating mutations as possible markers to identify everolimus responders (158).

Finally, Polo-like kinase 1 (Plk-1), a pro-mitotic protein, has been reported to be upregulated in 8% of MIBC cases published in the TCGA database and has been associated with bad prognosis in several other studies (159,160). An ongoing phase II trial focusing on the Plk-1 inhibitor volasertib showed only partial response in 14% of the enrolled patients (161). Similarly to what was previously remarked, this clinical trial looks poorly designed. All the enrolled patients had metastatic disease and already had a first round of conventional chemotherapy before being enrolled, but no information was available about the status of Plk-1.

Based on the TCGA study as well as on promising results published in several different types of cancer, there are many potential clinical trials that could still be tested for the treatment of UC. The RB pathways, as well as Aurora A kinase are some examples of potential targets that

could be further studied in a clinical setting. Many effective pharmaceutical tools are already available to us and the discovery of altered pathways in bladder cancer, as well as understanding the escape mechanisms employed by this disease to overcome our efforts to cure bladder cancer are the key to future success. One of the main efforts of this thesis is to further investigate such mechanisms and study new ways to make patients benefit from the drugs already available.

1.6 Summary and scope of the dissertation

Despite extensive research, the available therapeutic options for the treatment of bladder cancer are unfortunately limited to the ones we already had about 30 years ago. Targeted options for the treatment of this disease are desperately needed especially for advanced and metastatic stages. When I started this study, I had the firm belief that strong therapeutic tools were already in our hands but we just did not know how to properly use them. I had to decide what to target and how. The ultimate goal was to find a targeted therapy that ideally had low toxicity and was highly cytotoxic and specific for bladder cancer.

I decided to focus on the PI3K pathway because its relevance in bladder cancer has already been well characterized by numerous previous studies. The overall hypothesis was that by adequately blocking the PI3K pathway and its downstream targets it was going to be possible to induce cell death in a predicable sub-population of bladder cancer cell lines. To assess this hypothesis, I divided our study into 3 aims, which are addressed in chapter 3, 4 and 5 of this thesis:

1. **Aim 1: To discover predictive markers to assess the sensitivity of bladder cancer cell lines to small molecule inhibitors targeting the PI3K/AKT pathway.**

2. **Aim 2: To discover novel strategies to sensitize bladder cancer cell lines to small molecule inhibitors targeting the PI3K pathway.**
3. **Aim 3: To characterize the dependency of bladder cancer cell lines on more than one growth factor receptor regulating the PI3K pathway.**

This dissertation is based on 3 different manuscripts, one already published and 2 in preparation, each based on the characterization of the effects of a different small molecule inhibitor. Each paper addresses at least 2 of the 3 aims described above, sometimes overlapping each other. Table 1.3 presents the role of each publication in this thesis and its implications.

AKT inhibitor	*mTOR inhibitor*	*IGF-1R inhibitor*	
Predictability based upon genetic signature		Predictability based upon genetic signature	*Chapter 3*
Combination of small molecule inhibitor and conventional chemotherapy ↓ Block of autophagy as mechanism to overcome drug resistance	Combination of small molecule inhibitor and conventional chemotherapy ↓ Block of autophagy as mechanism to overcome drug resistance (investigated in greater depth) ↓ 4 arms xenograft study investigating the effects of mTORi in combination with autophagy blocker Chloroquine	Block of autophagy as mechanism to overcome drug resistance (investigated in greater depth) ↓	*Chapter 4*
		Investigation of the differential regulation of downstream pathways of IGF-1R dependent cells ↓ Evaluation of multiple growth factor receptor inhibitors as therapeutic treatment for bladder cancer	*Chapter 5*

Table 1.3: schematic representation of the content of the 3 publications this thesis is based on. Different parts of each manuscript fulfil the topics illustrated in each chapter of this dissertation.

Chapter 2. MATERIALS AND METHODS

This chapter is partially based upon "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" with the permission of the journal.

2.1 Materials and Methods for Chapter 3

2.1.1 Cell lines and culture conditions. The University of Michigan Urothelial Cancer (UMUC) panel of cell lines was acquired and genotyped by the Specimen Core of the MD Anderson Specialized Program of Research Excellence (SPORE) in Bladder Cancer. Cell lines were validated by short tandem repeat (STR) DNA fingerprinting using the AmpFλSTR Identifiler kit according to manufacturer instructions (Applied Biosystems). The STR profiles were compared with known American Type Culture Collection (ATCC) fingerprints (ATCC.org), to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/) (Nucleic Acids Research 37:D925-D932 PMCID: PMC2686526) and to the MD Anderson fingerprint database. The STR profiles matched known DNA fingerprints or were unique. The metastatic human urothelial cell line 253J B-V was generated in our laboratory from the 253J parental cell line by orthotopic "recycling" in nude mice (162). All cell lines were maintained at 37°C with 5% CO2 in modified Eagle's MEM supplemented with 10% fetal bovine serum (FBS), vitamins, sodium pyruvate, L-glutamine, penicillin, streptomycin and non-essential amino acids.

2.1.2 Reagents and antibodies. AZ7328, a competitive inhibitor of the AKT ATP-binding domain, was provided by AstraZeneca. The drug was reconstituted in dimethyl sulfoxide (DMSO) and stored at -20°C until use. The stock solution was diluted in medium just before use so that the DMSO concentration never exceeded 0.1%. BMS-754807 a pyrrolo[1,2-f][1,2,4]triazine based inhibitor of Insulin-like growth factor receptor (IGF-1R) (163) was provided by Bristol Mayer Squibb. The drug was dissolved in dimethyl sulfoxide (DMSO) and stored at -20 degrees Celsius. During each experiment the drug was diluted in media so that the total concentration of DMSO never exceeded 0.1%. Antibodies specific for IGF-1R β subunit,

phospho-AKT, AKT, cyclin D1, phospho-GSK-3β, GSK-3β, phospho- S6K1, S6K1, PTEN were purchased from Cell Signaling Technology, p27 from BD Biosciences, and β-actin from Sigma-Aldrich.

2.1.3 Detection of mutations by gene sequencing. A high-throughput approach was used by the MD Anderson Sequenome Core Facility to identify specific oncogene sequence-specific mutations on our panel of 12 bladder cancer cell lines (Table 3.2). Oligonucleotide primers (164) for amplifying gene coding exons were designed to give a product size in the range of 200 to 700 bp with a minimum of 40 bp flanking the splice sites using the Exon Primer program, which is bundled with the University of California at Santa Cruz Genome Browser (build hg17). M13F and M13R tags were added to the forward and reverse primers, respectively. Five nanograms of genomic DNA from each cell line were amplified in an 8-L PCR using AmpliTaq Gold (Applied Biosystems) on PE 9700 machines and subsequently cleaned using a diluted version of the EXO-SAP-based PCR product pre-sequencing kit (USB Corp.) dispensed by a nanoliter dispenser (Deerac Fluidics Equator by Promega). All PCR set-up procedures were performed in a 384-well format using a Biomek FX workstation after optimization. Sequencing reactions were then performed using the M13 primers along with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and cleaned with BET before separation on an ABI 3730xl DNA Analyzer. Base calling, quality assessment and assembly were performed using the Phred, Phrap, Polyphred, Consed software suite. All sequence variants identified were verified by manual inspection of the chromatograms. Mutation frequencies determined using this approach should be considered lower estimates as all exon sequences were not covered in all cases with perfect mutation capture. In contrast, the false-positive rate with this approach is low to nonexistent (165).

2.1.4 Evaluation of cell proliferation by MTT reduction. The acute and chronic effects of AZ7328 were measured at 48 and 120 h. In the 48 h assays 3–4 × 10_4 cells/well were plated in 96-well plates for 24 h in complete media with 10% FBS and then exposed to varying drug concentrations. After 48 h, cell proliferation was assessed by pulsing the cells for 2 h with dimethyl thiazolyl diphenyl tetrazolium salt (MTT) (5 mg/mL in PBS) followed by solubilization in 100 μL of DMSO. Longterm (120 h) assays were performed using a slightly modified version of a protocol published previously (166). Briefly, 500 cells/well were plated in 96-well plates for 24 h in complete media with 10% FBS and then exposed to varying drug concentrations. After 48 h the medium was removed, replaced with fresh medium containing the same concentrations of drug and cells were incubated for an additional 72 h. Cell proliferation was then assessed by pulsing the cells for 4 h with MTT (5 mg/mLin PBS) and solubilizing the reduced dye in 100 μL of DMSO. Color development was quantified by measuring the optical densities (ODs) at 570 nm and subtracting the ODs obtained at 660 nm (backgrounds). Results shown are mean ± SEM and were repeated in triplicate.

2.1.5 Western blot analysis. Cells were plated at approximately 70–80% confluence on 10 cm dishes and exposed to AZ7328 for the times indicated. The cells were harvested and disrupted on ice in lysis buffer [50 mmol/L TRIS-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 25 mmol/L NaF, 1% NP40, 0.1% Triton-X100, 0.1 mmol/L Na_3VO_4, 12.5 mmol/L β-glycerophosphate, 1 mmol/L PMSF, complete protease inhibitor cocktail (Roche)]. Protein concentrations were determined using the Bio-Rad Bradford protein assay reagent (Bio-Rad Laboratories). Approximately 50–60 μg of each protein sample was boiled for five minutes and separated by 15% SDS PAGE at 150 mV in electrophoresis buffer [25 mmol/L TRIS-HCl (pH 8.3), 192 mmol/L glycine, 0.1% SDS]. Proteins in the gels were electrophoretically transferred

onto nitrocellulose membranes in transfer buffer (25 mmol/L TRIS-HCl, 192 mmol/L glycine, 20% methanol) for 16 h at 40 mV and 4°C. The membranes were washed in PBS with 0.1% Tween 20 (PBS-T), blocked in 5% nonfat dry milk for 30 min at room temperature with shaking and then rinsed with PBS-T. The membranes were incubated with primary antibodies diluted 1:1,000 in PBS-T containing 5% milk overnight, followed, after further washing, by incubation for 1 h at room temperature in horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology) diluted in PBS-T containing 5% milk (anti-rabbit 1:3,000, anti-mouse 1:5,000). The probed proteins were detected using the enhanced chemiluminescence system (GE Healthcare/Amersham Biosciences) according to the manufacturer's instructions.

2.2 Materials and Methods for Chapter 4

2.2.1 Reagents and antibodies. AZ12594264-012 and AZD8055 (167-169), a competitive inhibitor of the mTOR ATP-binding domain, was provided by AstraZeneca (Alderley Park, Macclesfield, Cheshire, United Kingdom). The drug was reconstituted in dimethyl sulfoxide (DMSO) and stored at -200C until use. The stock solution was diluted in medium just before use, so that the DMSO concentration never exceeded 0.5%. Recombinant human (rhTRAIL) was purchased from R&D Systems, gemcitabine from Eli Lilly and Co., cisplatin from Teva Parenteral Medicines, Inc., rapamycin and Chloroquine disolphate salt from Sigma-Aldrich. Antibodies specific for LC3 were purchased from BD Biosciences and β-actin from Sigma-Aldrich. The Lipofectamine RNAiMAX was purchased from Invitrogen. The RT-PCR primers were purchased from ABI.

2.2.2 Cell cycle analysis. 9–12 × 10^4 cells were plated in 6-well plates for 24 h in complete media with 10% FBS and then exposed to varying drug concentrations. After 24 h the cells were trypsinized and pelleted by centrifugation. The cells were re-suspended in PBS containing 50

μg/mL of propidium iodide (PI), 0.1% Triton X-100 and 0.1% sodium citrate. PI fluorescence was measured by fluorescence-activated cell sorting analysis (FACS) (FL-3 channel, Becton Dickinson). Cells displaying a hypodiploid content of DNA, indicative of DNA fragmentation, were considered apoptotic. All conditions were replicated in triplicate.

2.2.3 Quantification of DNA synthesis by 3H incorporation. 6–8 × 10^3 cells per well were plated in 96-well plates for 24 h in complete media with 10% FBS and then exposed to varying drug concentrations. After 24 h medium was removed and replaced with fresh cell culture medium containing 10% FBS and 10 μCi/mL [$_3$H] thymidine (MP Biomedicals). The cells were pulsed with [$_3$H] thymidine for two hours and the media was subsequently removed. Cells were then lysed by the addition of 0.1 mol/L KOH and harvested onto fiberglass filters using a cell harvester (Perkin Elmer/Wallac). The incorporated tritium was quantified in a 1450 MICROBETA Trilux liquid scintillation and luminescence counter (PerkinElmer Life Sciences). Results shown are mean ± SEM, n = 8.

2.2.4 Analysis of autophagy by anti-LC3 immunofluorescence. Twenty thousand cells were seeded in 8-well chamber slides (Becton, Dickinson and Co.) for 24 h in complete medium with 10% FBS and then exposed to various drug concentrations. After 24 h, the medium was removed and the cells were washed with PBS, fixed in 4% paraformaldehyde diluted in PBS and permeabilized in 100 μg/ml of digitonin diluted in PBS. The cells were then blocked in a solution of 5% horse serum and 1% goat serum diluted in PBS followed by overnight incubation at 4°C with mouse antibody against LC-3 (MBL International) diluted 1:300 in blocking buffer. After 24 h, the cells were washed in PBS, and incubated for 1 h with anti-mouse antibody DyLight 549 (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:1,000 in blocking buffer. After PBS washing the cells were counterstained with Sytox Green (Life technologies, Grand

Island, NY) diluted 1:10,000 in PBS. The cells were then observed under a fluorescence microscope (ZEISS Axioplan2, Carl Zeiss Microscopy, LLC). Pictures were taken for each condition using an ORCA-ER Hamamatsu camera (Hamamatsu Photonics, K.K.) and Image-Pro Plus 5.1 imaging software (Media Cybernetics, Inc.).

2.2.5 Soft agar cultures. 6 well plates were filled with a bottom layer of 0.5 agarose gel diluted in complete media with 10% FBS. 10000 cells/well were diluted in 0.3% agarose gel diluted in complete media with 10% FBS containing different drug concentrations and plated over the bottom layer in 6 well plates. The cells were incubated for 2 weeks until colonies were observed. The colonies were then photographed by microscope (Olympus IX81, Olympus America Inc., Center Valley, PA) and their average diameter was calculated analyzing 10 colonies for each of the replicates (the experiments have been done in triplicates) by Slidebook 4.2 (Intelligent Imaging Inc., Denver, CO).

2.2.6 Gene silencing. Briefly, cells were seeded in 24 well plates for cell cycle analysis and in 10cm dishes for immunoblot and RT-PCR purposes. They were seeded at 60-70% confluence in the presence of 25nM of appropriate siRNA and Lipofectamine^{TM} RNAiMAX according to the reverse transfection protocol provided by the manufacturer. Cells were then incubated with the siRNA for 48h to allow optimal gene silencing. Media was then replenished and cells in 24-well plates were exposed to various concentrations of BMS-754807. After 48 additional hours, cells from 10cm dishes were collected and processed as described in the immunoblotting and RT-PCR section, while cells seeded in 24-well plates and exposed to the IGF-1R inhibitor were processed as described in the cell cycle section.

2.2.7 RNA isolation and Real-time reverse transcription PCR.

Similarly to what we previously described (170), RNA was isolated using Trizol according to the manufacturer's protocol. Taqman-based Real-Time PCR was then used to quantify gene expression, loading 20ng of RNA on 96-well plates. The comparative ΔΔCt method was used to estimate gene expression and the data were plotted as relative quantity (CQ) ± min and max. PPIA was used as an endogenous control. All the reactions were run on the StepOne Plus PCR system (ABI).

2.2.8 Xenograft study

6-8 weeks old female athymic nude mice were purchased from the National Cancer Institute and kept under aseptic conditions in the South Campus Animal core facility at The MD Anderson Cancer Center at Houston, Texas. The facility was certified by the American Association for Accreditation of Laboratory Animal Care and in agreement with current regulations and standards of the U.S. Department of Health and Human Services, the U.S. Department of Agriculture, and the NIH. The human urothelial cancer cells UM-UC-14 were transduced with a lentiviral vector encoding luciferase (luc) and red fluorescent protein (RFP; mCherry) as previously described (171). Cells were then sorted out of their RFP expression by Fluorescence Activated Cell Sorting (FACS) using an Influx High-Speed sorter (BD Biosciences). Luciferase activity was quantified in vitro using D-luciferin (150 μg/mL) and the IVIS bioluminescence system (Xenogen Co.) Tumor cells were grown to confluence in 150mm culture disches and then collected by trypsinization, centrifuged at 1500 rpm for 10 min and resuspended in 10% FBS MEM at the concentration of 2.4 million cells/mL. 50 μL of cell suspension equal to 120.000 cells were then injected orthotopically into the bladder wall by lower laparotomy. Tumors were allowed to grow for 4 days before starting any therapy. Mice were then treated

with 50 mg/Kg of Chloroquine 3 times/week via IP injection and with 20 mg/Kg of AZD8055 daily administered by oral gavage according to the experiment plan. Tumor growth was quantified by Ivis Spectrum imaging system. After 28 days mice were sacrificed and tumors were harvested and preserved under OPT.

2.2.9 Ki67 staining

OPT was removed and samples were rehydrated in PBS. DIVA from Thermo/Lab Vision was used as antigen retrieval and incubated for 20min. Samples were then blocked with 3% H_2O_2 and blocked with a solution of 5% horse serum and 1% goat serum. Anti Ki67 antibody from Sigma Aldrich was then added to the samples and incubated overnight. The next day, the samples were washed and blocked again with the same solution and then incubated with secondary HRP-conjugated antibody for 1h at room temperature. The slides were then washed and incubated with DAB for about 11 minutes and then counterstained with Gill's hematoxylin for 14 seconds.

2.3 Materials and Methods for Chapter 5

2.3.1 Antibodies.

Specific antibodies for IGF-1R β subunit, phospho-AKT, AKT, phospho-ERK, ERK, phospho-S6K and S6K were purchased from Cell Signaling Technology

2.3.2 Immunoblotting.

Cells were plated at 70-80% confluence in 10cm dishes and exposed at various concentrations of BMS-754807 for 24h. The western blots were performed as previously described (164). The probed proteins were detected by using the Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate (Perkin Elmer).

CHAPTER 3. PREDICTABILITY OF THE EFFECTS OF SMALL MOLECULE INHIBITORS TARGETING THE PI3K PATHWAY BASED ON MUTATION STATUS AND PROTEIN EXPRESSION

This chapter is partially based upon "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" with the permission of the journal.

3.1 Characterization of activation oncogenic mutations in the PI3K/AKT pathway in bladder cancer cells

Components of the PI3K/AKT/mTOR pathway are mutated in a large subset of bladder cancers, (11) making activated AKT an attractive candidate for therapeutic target in the disease. I hypothesized that any alteration responsible for the activation of the PI3K/AKT/mTOR pathway (i.e., activating mutations in growth factors receptors, PIK3CA, and/or Ras or inactivating mutations in PTEN and/or TSC1/2) would make bladder cancer cells sensitive to small molecule inhibitors targeting the PI3K/AKT/mTOR pathway.

To test this hypothesis, I examined the effects of 3 different small molecule inhibitors: the AKT inhibitor AZ7328, the TORC1+2 inhibitors AZ4264/AZD8055 and the IGF-1R inhibitor BMS-754807. I specifically selected these 3 drugs because of the positive results reported in other types of cancer. Several AKT inhibitors were reported to have good antitumor and even pro-apoptotic activities in a wide range of cancer cell lines like glioblastoma, endometrial, prostatic, ovarian and breast cancer cells, particularly if overexpressing AKT (172-174). AZD8055 was selected for its unique capability to inhibit mTORC1 and mTORC2 at the same time, in contrast with most commercially available mTOR inhibitors like RAD001 and rapamycin that only target mTORC1. At the time I started working with AZD8055, there were already very promising publications vouching for the outstanding growth inhibitory effects as well as pro-apoptotic properties of this compound (167,168). A good record of publications is also available for the IGF-1R inhibitor BMS-754807. This IR/IGF-1R inhibitor has been shown to be a potent anti-tumor drug in several different kinds of cancers with pro apoptotic effects in rhabdomyosarcoma (163,175). Being a potential regulator of the PI3K pathway, working with BMS-754807 represented a good opportunity to study the effects of RTK inhibitions on bladder

cancer cells with and without genetic alteration in the PI3K pathway and compare them to determine the importance of growth factor receptors in these two subpopulations.

In the beginning of the study, I expected to observe consistent patterns of sensitivity and resistance when comparing the spectrum of sensitivity of the two AstraZeneca compounds targeting AKT and mTOR across the panel of bladder cancer cell lines. It was more difficult to anticipate the effects of the IGF-1R inhibitor on bladder cancer cells. Like many growth factor receptors, IGF-1R regulates many different downstream pathways like the PI3K and MAPK pathway. The possibility that the pattern of resistance for this molecule was different from the ones identified for the former compounds was plausible. Similarly, predicting whether molecular alterations in the PI3K/AKT pathway would lead to enhanced sensitivity to this inhibitor was difficult, because of the presence of other growth factor receptors that could potentially compensate the inhibition of IGF-1 receptors. Another factor to be considered was that the presence of activating mutations downstream the IGF-1R could wean bladder cancer cells from the necessity to rely on RTK activation, greatly attenuating the effects of BMS-754807. The first step to link genetic signature with pattern of sensitivity to our selected drugs was to characterized a subset of 12 molecularly diverse human bladder cancer cell lines for the presence of specific activating oncogene mutations and inactivation mutations of tumor suppressors (Table 3.1) in comparison with the mutations shown in Table 3.2.

	EGFR	FGFR3	c-MET	PIK3CA	RAS	AKT1	AKT2	AKT3
253J B-V				E545G				
J82		K652E K560E		P124L				
T24			T1010L		Hras-G12V			
UM-UC-3				E545K	Kras-G12C			
UM-UC-5	Amplified			E545K				
UM-UC-6		S249C	T1010L	E545K				
UM-UC-9								
UM-UC-10								
UM-UC-11								
UM-UC-13								
UM-UC-14		S249C						
UM-UC-16		S249C		H1047R				

Highlighted mutations include EGFR, FGFR3, c-MET, PIK3CA, RAS and AKT, 1/2/3.

Table 3.1 Summary of PI3K/AKT pathway activating oncogenic mutations in a panel of 12 bladder cancer cell lines Adapted with permission from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

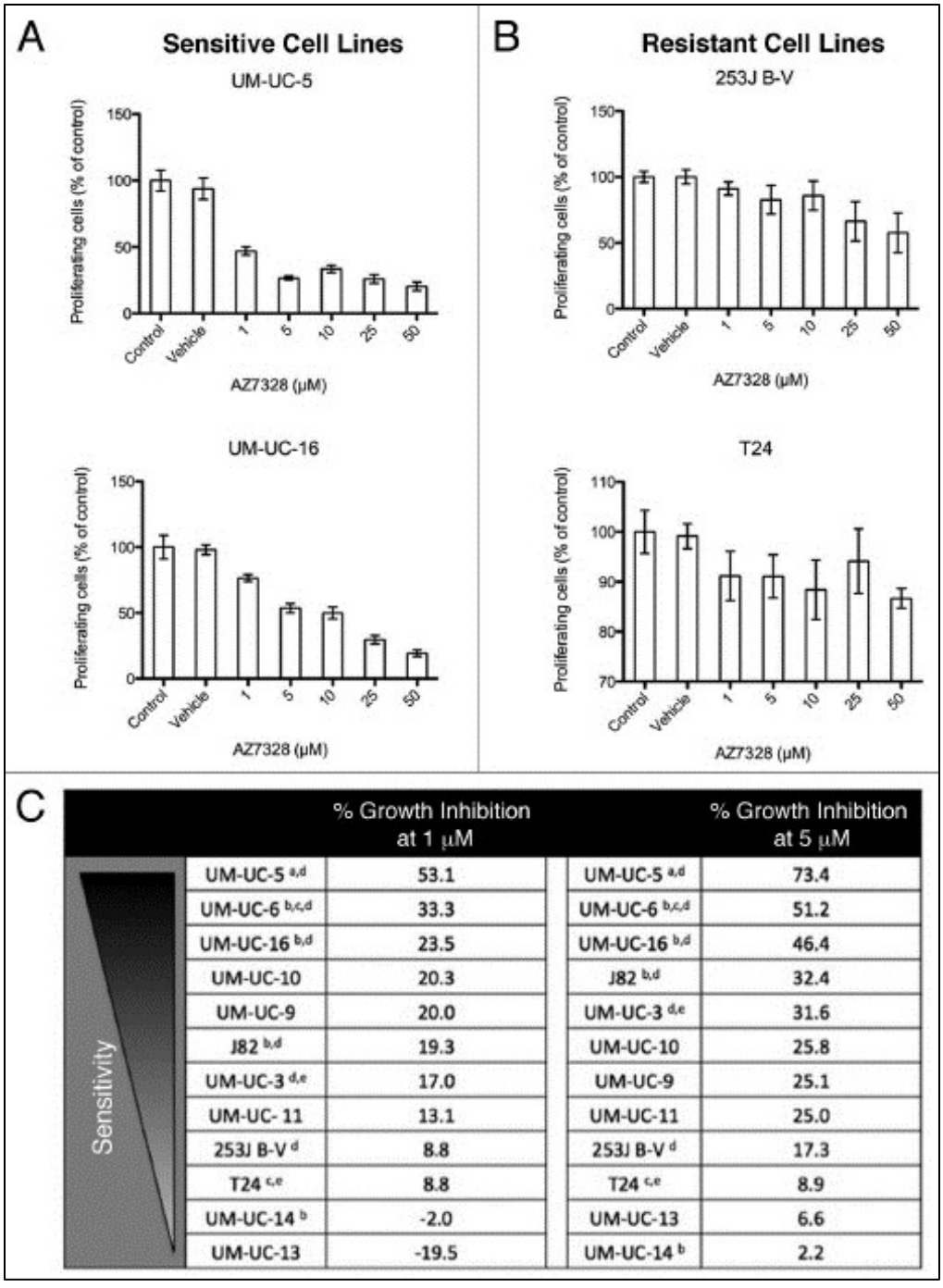
Our gene sequencing results were cross-referenced with those of the COSMIC (Catalog of Somatic Mutations in Cancer) cancer database for accuracy and all differences were reconciled. Two thirds of the cell lines (8/12) possessed at least one molecular defect that would be expected to promote PI3K/AKT/mTOR pathway activation and six contained multiple activating mutations. The following molecular alterations were found: EGFR amplification (UM-UC-5) (176), FGFR3 point mutation (J82, UM-UC-6, UM-UC-14, UM-UC-16), c-MET point mutation (T24, UM-UC-6), PIK3CA alteration (253J BV, J82, UM-UC-3, UM-UC-5, UM-UC-6, UM-UC-16) and H or K-Ras mutations (T24 and UM-UC-3). There were no activating AKT mutations in our panel of cells.

AKT1	V600-1798G-1	Q227-C651	G12-34G	T354M-C1061T	Y1021C-3062
E17K-G49A	V600-1799T-1	R201-C601	G12-35G	**PIK3CA**	**PIK3R1**
G173R-G517C	V600-1799T-2	**HIF1A**	G13-37G	A1046V	D560Y
K179M-A536T	V600-1800G	Q697E-C2089G	G13-38G	C420R	G376R
AKT2	**CDK4**	**IDH1**	Q61-181C	E110K	G376R_2
G175R-G523C	R24C-C70T-2	R132-C394T	Q61-182A	E418K	intron1
AKT3	R24H-G71A	R132-G295T	Q61-183A	E453K	intron2
E17K-G49K	**CTNNB1**	R172-A514T	**MEK1**	E542-1624G	M326I-G978
G171R-G511A	D32-94G	**IDH2**	D67N-G119A	E542-1625A	N564K
ALK	D32-95A	R172-G515	H1112-3335	E545-1633G	**PRKAG1**
1174I-T3520A	G34-101G		H1112Y-C3334T	E545-1634A	R70Q
A877S-G2G29T	S33-97T	**IGF1R**	M1268T-T3803C	E545-1635G	**PRKAG2**
D1091N-G3271A	S37-109T	A1347V-C40404T	N375S	F909L	N488I
F1245C-T3734G	S37-110C	**JAK2**	N848S	G1049R	**RET**
F1245V-T3734G	S45-133T	V617F-G1849T	R988C	H1047	M918T
I1171N-T3512A	S45-134C	**KIT**	T1010I-C3029T	H1047-1	**Rictor**
I150T-T3749C	T41-121A	D816H-GC	Y1248-T3742	H1047Y	M675I-G2025A
L560F-G1680C	**EGFR**	D816V-AT	Y1248C-A3743G	H701P	**STK11**
M1166R-T3497G	K860I-A2579T	K642E-AG	Y1253D-T3757G	K111N	D194-A591T
R1275Q	L858R-TG	L576P-TC	**NRAS**	M1043I-G3129	D194-G590
BRAF	S720P-T2158C	N556D-AG	G12-G34	N345K	F354L-C1062G
D594-1781A	T790M-C2369T SPLIC	R634W-CT	G12-G35	P539R	P281L-C842T
E586K-1756GA SPLICE	T854I-C2561T	V559-T	G13-G37	Q060K	**TNK2**
G466-1397G	Y813C-A2438G	V560D-TA SPLIC	G13-G38	Q546-1636C	E346K-G1036A
G466R-1396-GC	**Era**	V825A-TC	Q61-A182	Q546-1637A1	R99Q-G296A
G469-1407A	G400V	Y553N-TA	Q61-A183	R088Q	
K601E AG	**FRAP**	**KRAS**	Q61-C181	S405F	
K601N A	M135T-T404C	A146-436	**PDPK1**	T1025-3073A	
L597R-1790TG	**GNAS**	G10R	D527E-C1581G	Y1021-3061T	

Table 3.2: Panel of activating oncogenic mutations assayed at the MD Anderson Sequenome Core Facility. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal

3.2 Range of sensitivity to AZ7328 in bladder cancer cells

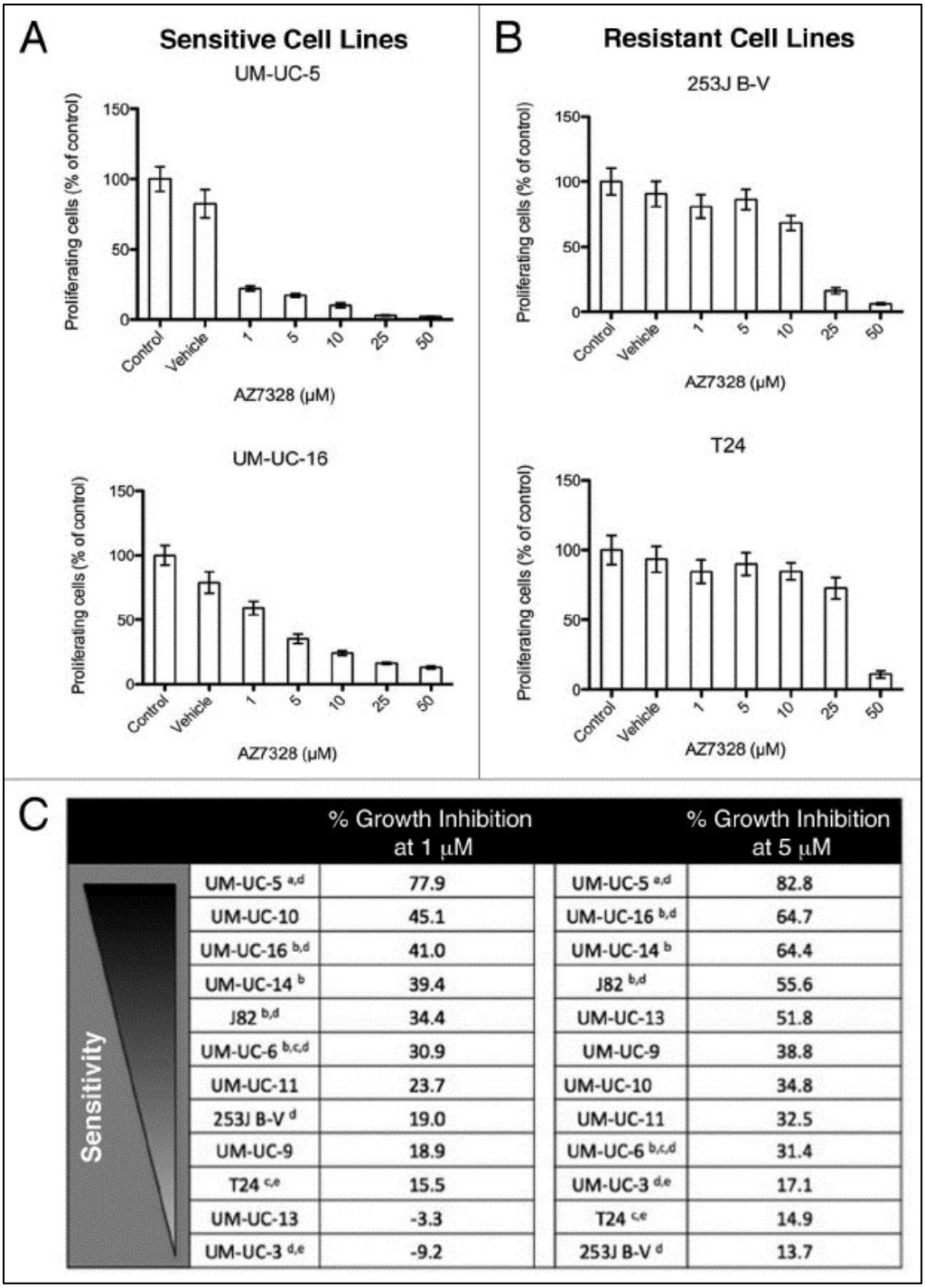
I collaborated with a postdoctoral fellow (Rian Dickstein) to examine the effects of the AstraZeneca AKT inhibitor on the cell lines. We exposed the cells to increasing concentrations of AZ7328 and measured cell proliferation at 48 and 120 h by MTT reduction (Fig. 3.1A and B and Fig. 3.2A and B). We rank ordered the cell lines by sensitivity to AZ7328 at 1 and 5 μM drug concentrations (Figs. 3.1C and 3.2C). We extended these observations in one cell line that was on the sensitive end of the spectrum (UM-UC-5) and one on the resistant end (T24) to confirm proliferative inhibition measured by a reduction in DNA synthesis using a [3H] thymidine incorporation assay (Fig. 3.3). All of the cell lines that were relatively more sensitive to AZ7328 at 48 h had an activating PIK3CA mutation (UM-UC-5, UM-UC-6, UM-UC-16). However, there was variability in the PI3K/AKT/mTOR pathway alterations among those cell lines that were more resistant to AZ7328 at 48 h, yielding an unpredictable pattern of resistance. With longer drug exposure (120 h), the sensitive (UM-UC-5 and UM-UC-6) and resistant (253J B-V and T24) relationships remained generally constant.



C

	% Growth Inhibition at 1 μM			% Growth Inhibition at 5 μM
Sensitivity	UM-UC-5 [a,d]	53.1	UM-UC-5 [a,d]	73.4
	UM-UC-6 [b,c,d]	33.3	UM-UC-6 [b,c,d]	51.2
	UM-UC-16 [b,d]	23.5	UM-UC-16 [b,d]	46.4
	UM-UC-10	20.3	J82 [b,d]	32.4
	UM-UC-9	20.0	UM-UC-3 [d,e]	31.6
	J82 [b,d]	19.3	UM-UC-10	25.8
	UM-UC-3 [d,e]	17.0	UM-UC-9	25.1
	UM-UC- 11	13.1	UM-UC-11	25.0
	253J B-V [d]	8.8	253J B-V [d]	17.3
	T24 [c,e]	8.8	T24 [c,e]	8.9
	UM-UC-14 [b]	-2.0	UM-UC-13	6.6
	UM-UC-13	-19.5	UM-UC-14 [b]	2.2

Figure 3.1: Sensitivity of bladder cancer cell lines to increasing concentrations of AZ7328 as measured in 48 h MTT assays.(**A**) The anti-proliferative effects of AZ7328 in two relatively sensitive cell lines (UM-UC-5 and UM-UC-16). (**B**) The anti-proliferative effects of AZ7328 in two relatively resistant cell lines (253J B-V and T24). Note the differences in scales. (**C**) Rank ordering of sensitivity to AZ7328 at 48 h of exposure in a panel of 12 bladder cancer cell lines by the percentage of proliferative inhibition induced at both 1 and 5 μM concentrations. a EGFR amplification, b FGFR3 mutation, c c-MET mutation, d PIK3CA mutation, e RAS mutation. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.



	% Growth Inhibition at 1 μM		% Growth Inhibition at 5 μM
UM-UC-5 [a,d]	77.9	UM-UC-5 [a,d]	82.8
UM-UC-10	45.1	UM-UC-16 [b,d]	64.7
UM-UC-16 [b,d]	41.0	UM-UC-14 [b]	64.4
UM-UC-14 [b]	39.4	J82 [b,d]	55.6
J82 [b,d]	34.4	UM-UC-13	51.8
UM-UC-6 [b,c,d]	30.9	UM-UC-9	38.8
UM-UC-11	23.7	UM-UC-10	34.8
253J B-V [d]	19.0	UM-UC-11	32.5
UM-UC-9	18.9	UM-UC-6 [b,c,d]	31.4
T24 [c,e]	15.5	UM-UC-3 [d,e]	17.1
UM-UC-13	-3.3	T24 [c,e]	14.9
UM-UC-3 [d,e]	-9.2	253J B-V [d]	13.7

Figure 3.2: Sensitivity of bladder cancer cell lines to increasing concentrations of AZ7328 as measured in a 120 h MTT assay. (**A**) The anti-proliferative effects of AZ7328 in two relatively sensitive cell lines (UM-UC-5 and UM-UC-16). (**B**) The anti-proliferative effects of AZ7328 in two relatively resistant cell lines (253J B-V and T24). (**C**) Rank ordering of sensitivity to AZ7328 at 120 h of exposure in a panel of 12 bladder cancer cell lines by the percentage of proliferative inhibition induced at both 1 and 5 μM concentrations. aEGFR amplification, bFGFR3 mutation, cc-MET mutation, dPIK3CA mutation, eRAS mutation. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

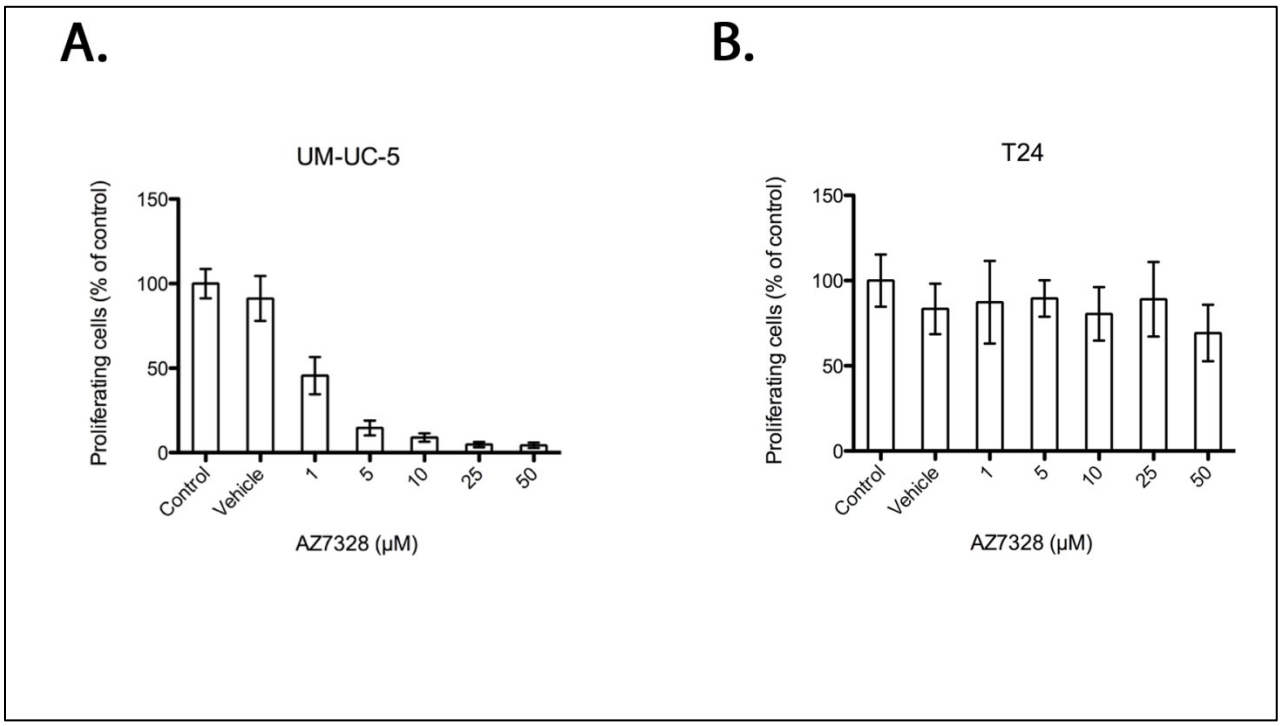


Figure 3.3: Sensitivity of urothelial carcinoma cell lines to increasing concentrations of AZ7328 as measured by a 24 h [3H] thymidine incorporation assay. A, The anti-proliferative effects of AZ7328 in one sensitive cell lines (UM-UC-5). B, The anti-proliferative effects of AZ7328 in one resistant cell lines (T24). Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

3.3 On target effects

To confirm that AZ7328 blocked AKT, we assessed the status of phospho-AKT and downstream targets in cell lines throughout the spectrum of sensitivity. We chose to evaluate several components of the PI3K/AKT/mTOR pathway: ribosomal p70s6 kinase 1 (or S6K1, which controls protein synthesis and cell growth), GSK-3β (which controls cell metabolism), cyclin D1 (a cell cycle progression protein), and p27 (a cyclin dependent kinase inhibitor) (177-179). We measured drug effects at the 1–3 h time point because time course studies indicated maximal drug effect within this range (Fig. 3.4). Our first dose-response experiments confirmed that AZ7328 reduced phosphorylation of S6K1 and GSK-3β to a similar extent at corresponding concentrations in all the cell lines assessed (Fig. 3.5). We also observed a strong concentration-

dependent increase in the levels of phosphorylated AKT (at both phosphorylation sites) consistent with prior findings showing that ATP-competitive inhibitors of AKT hold the protein in a hyperphosphorylated but catalytically inactive form (Fig. 3.6) (180).

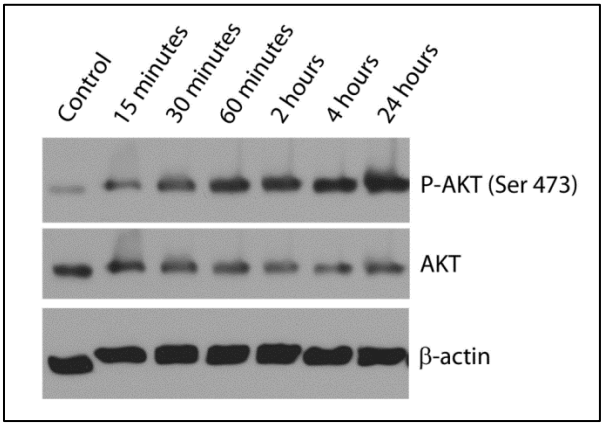


Figure 3.4: Time dependent effects of AKT inhibition with AZ7328 in UM-UC-3 as assessed by phosphorylation of AKT at Ser 473. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

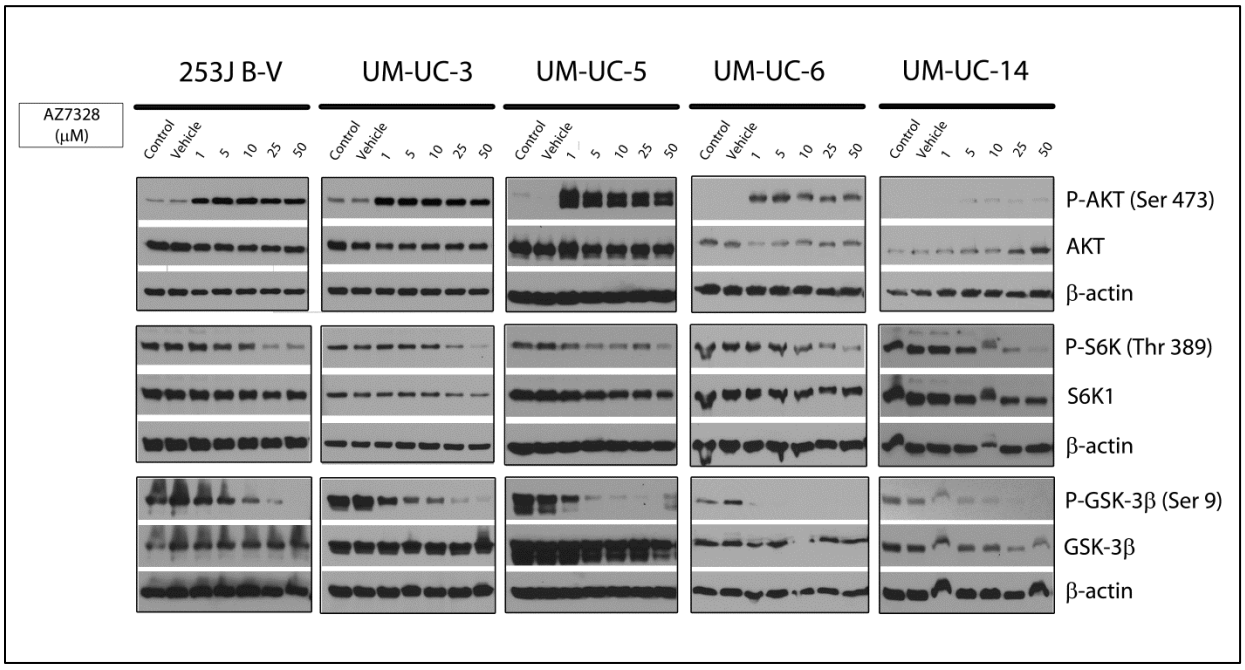


Figure 3.5: Pharmacodynamic response of selected cell lines to AZ7328. Each column represents a dose response assessment of the following markers within a cell line (immunoblotting with phospho-AKT, phospho-S6K1 and phospho-GSK-3β). Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

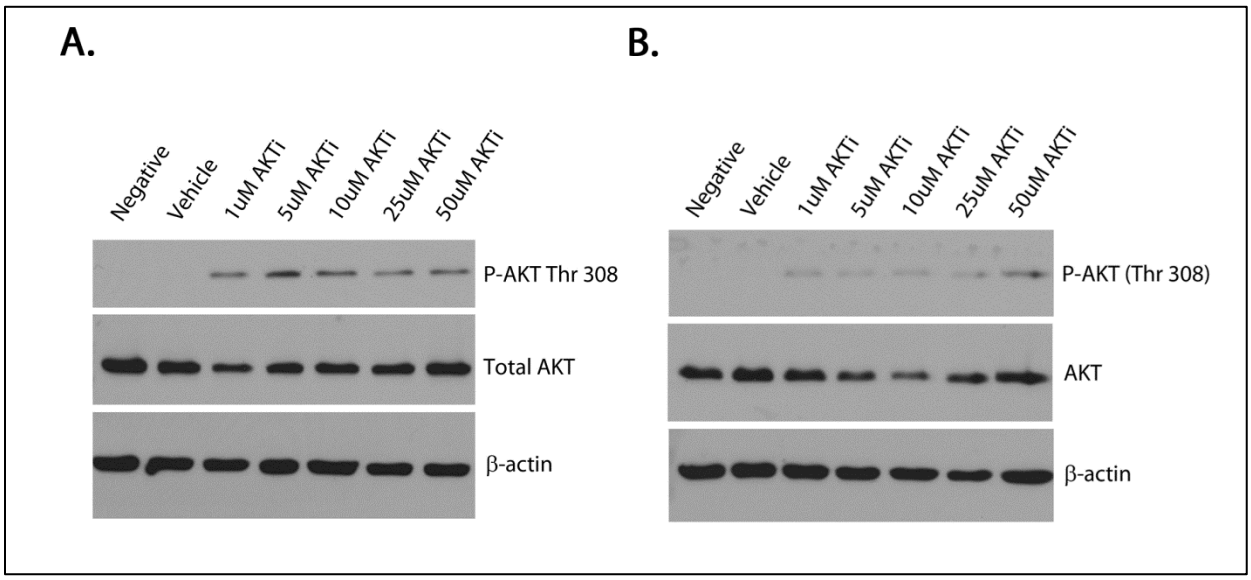


Figure 3.6: Pharmacodynamic response of selected cell lines to AZ7328. A, Western blot analysis of a dose response treatment for UM-UC-3 as assessed by phosphorylation of AKT at Thr 308. B, Western blot analysis of a dose response treatment for 253J B-V as assessed by phosphorylation of AKT at Thr 308. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

We then went on to confirm the on-target effects of AZ7328 by demonstrating a dose-dependent decrease in the downstream molecule cyclin D1 in UC5 but not in T24. Unfortunately, the western blot for p27 is inconclusive, as no clear dose dependent effect can be observed in T24, while there seems to be an increase in UC5 cells. However, a lower exposure would be needed to clearly address this issue. (Fig. 3.7). Collectively, our results showed that the effects of the drug on substrate phosphorylation were consistent across the cell lines in spite of the observed differences in their sensitivities to AZ7328-induced proliferative inhibition. Although PTEN deletion is a relatively rare event, reduced PTEN expression is found in a majority of muscle-invasive bladder cancers (181). Therefore, in addition to characterizing cell lines for the presence of inactivating mutations, we also measured PTEN protein levels by immunoblotting.

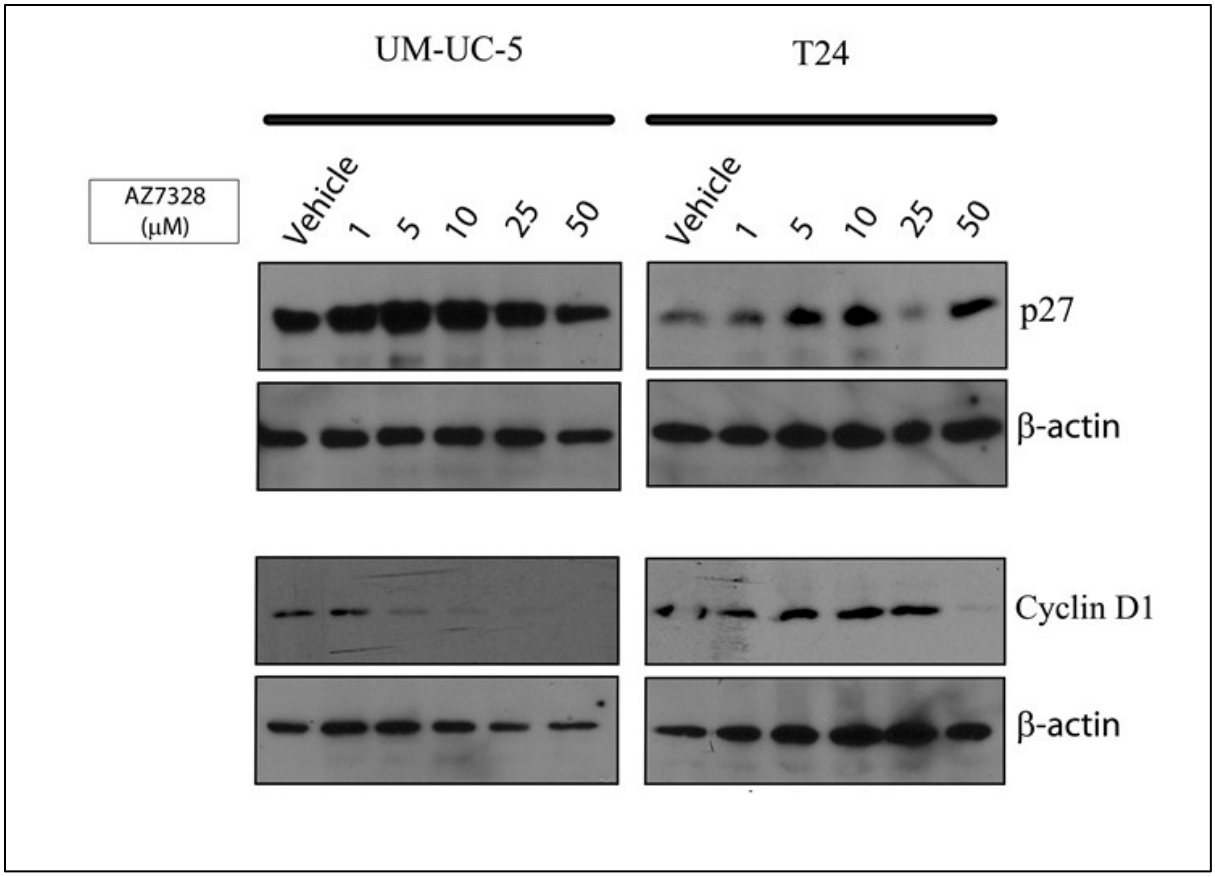


Figure 3.7: Dose-dependent effects of AZ7328 on Cyclin D1 and p27. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

Among the 12 cell lines tested, we found PTEN to be completely absent in two (J82 and UM-UC-3), but there was a 50% decrease in the amount of PTEN in three additional cell lines (UM-UC-9, UM-UC-10 and UM-UC-16) (Fig. 3.8A). Of these cell lines, UM-UC-16 displayed the greatest relative sensitivity to AKT inhibition, followed by J82, UM-UC-9, and UM-UC-10. Given the variability in PTEN expression among the responders and non-responders it is unreliable to assign any predictive ability of PTEN status to AZ7328 sensitivity. AKT phosphorylation is controlled by many upstream inputs (182). Therefore, we also compared baseline AKT phosphorylation levels across our panel as a more direct measure of AKT activity (Fig. 3.8B). Interestingly, high basal AKT phosphorylation was not associated with either

PIK3CA/PTEN status or sensitivity to AKT inhibition. For example, UM-UC-11 and UM-UC-13 cells (neither of which have PI3K/AKT/mTOR pathway activation) expressed high basal phospho-AKT levels despite being relatively resistant to AZ7328. Conversely, UM-UC-5 and UM-UC-6 cells expressed relatively low phospho-AKT levels even though they both contain activating PIK3CA mutations and were more sensitive to AKT inhibition. These discrepancies might be due to the presence of additional cell alterations that we did not take in account during our sequencing. Awhole genome sequence approach may help to better address this issue.. Irrespective of the mechanisms involved, the data clearly show that levels of phospho-AKT expression are also not predictive of sensitivity to AKT inhibition in bladder cancer cells. TSC is a syndrome resulting from the loss of two autosomal dominant genes that produce harmartin (TSC1) and tuberin (TSC2) (183,184). Functionally, the TSC1/TSC2 complex is downstream of AKT where TSC2 is directly phosphorylated and inactivated by AKT. TSC activation attenuates mTOR via a specific GTPase activating protein activity of TSC2 toward RAS homolog enriched in brain (RHEB) (185). Deletions of the long arm of chromosome 9 are the most common genetic alterations in urothelial carcinoma and mapping studies have linked this with the TSC1 locus (186). Other studies have found TSC1 mutations in 12% of bladder tumors. These results indicate that TSC1 mutations may play a role in the development of many sporadic bladder tumors (187). We screened our panel of cell lines for the level of TSC1 protein expression and found a varying range; however, these differences did not correlate with sensitivity to mTOR inhibition with rapamycin (Fig. 3.9). However, a deeper study would be needed to make strong conclusions about this possibility. A western blot analysis addressing the expression of TSC2 as well as TSC1/2 DNA sequencing to detect activating mutations would allow us to draw conclusions in a more confident way.

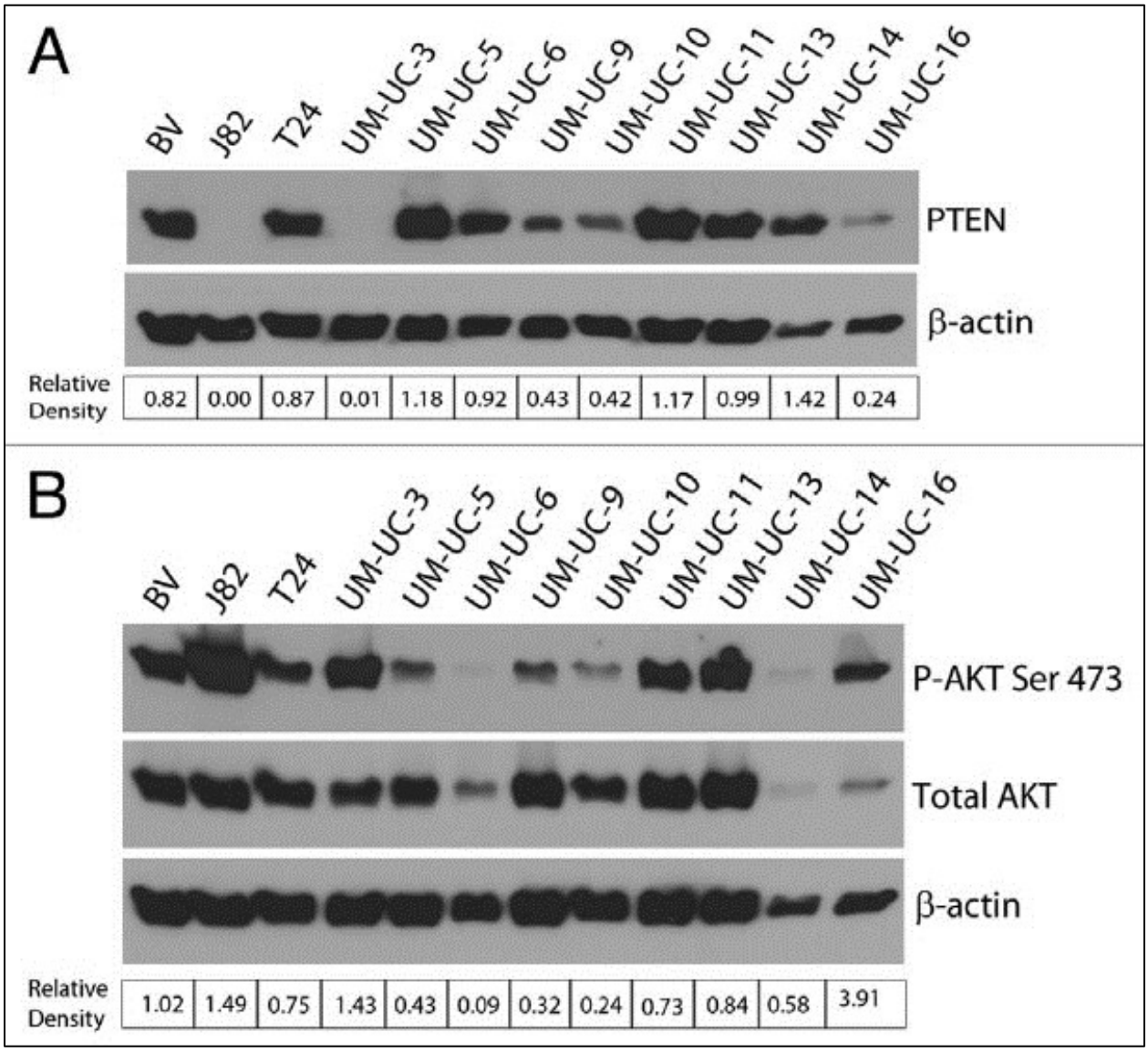


Figure 3.8: Potential predictors of response to AKT inhibition. (A) Western blot analysis of baseline PTEN status among the panel of 12 cell lines. The corresponding relative density indicates the PTEN band intensity relative to β-actin. (B) Western blot analysis of baseline AKT Ser 473 phosphorylation status among the panel of 12 cell lines. The corresponding relative densities indicate the phospho-AKT band intensity relative to total AKT. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal

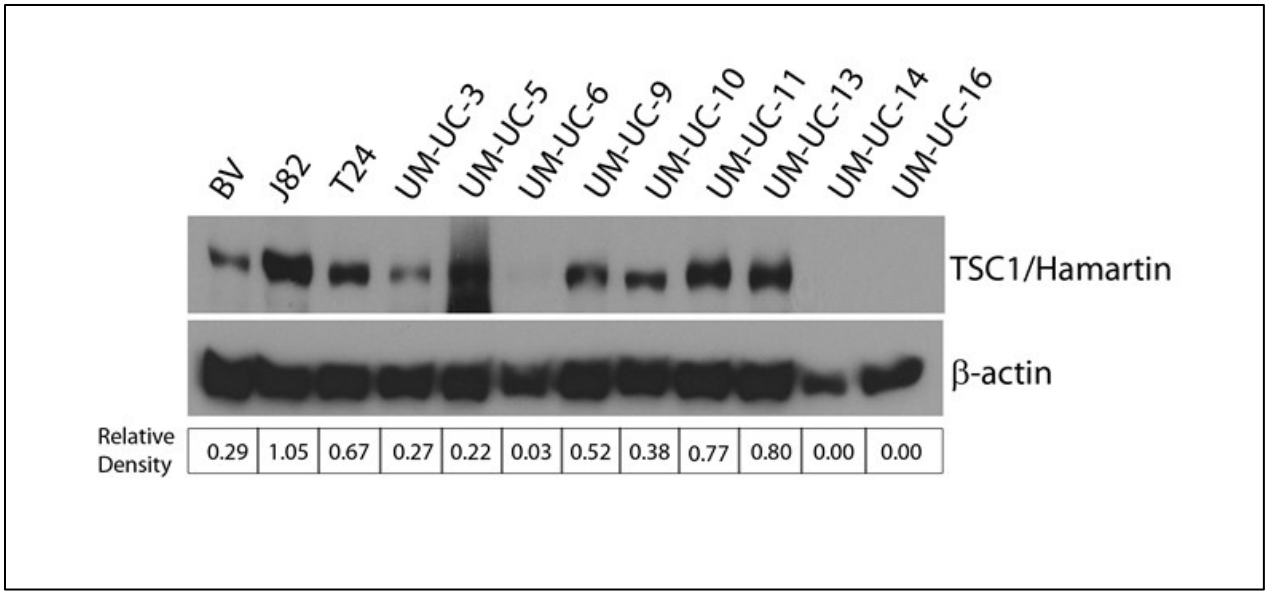


Figure 3.9: Western blot analysis of baseline TSC1/Hamartin status among the panel of 12 cell lines. The corresponding relative density indicates the TSC1/Hamartin band intensity relative to β-actin. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

3.4 Correlation between IGF-1R expression and sensitivity to IGF-1 receptor inhibitor BMS-754807

I tested the effects of BMS-754807 on proliferation on a panel of 30 bladder cancer cell lines. Cells were grown in 10% FBS MEM media, to activate the IGF-1R, and then exposed to increasing concentrations of the inhibitor (0, 0.01-50 μM) to determine the IC50 values using MTT assays (Fig.3.10A). Under these conditions it was possible to observe a wide range of sensitivity that I divided into 3 different phenotypes: resistant (IC50>2 μM), intermediate (2 μM>IC50>1 μM) and sensitive (IC50<1 μM). This discrimination was made accordingly to previously published data (175). Carboni *et al.* showed how BMS-754807's IC50s spread across a wide range of concentrations over 21 types of cancers (excluding bladder cancer). In this study all the sensitive cell lines were inhibited at submicromolar concentrations, ranking rhabdomyosarcoma, HNSCC, NSCLC, liposarcoma and neuroblastoma the most sensitive types

of cancer. One of the most remarkable observations that I would like to report, is that in each type of cancer, Carboni *et al.* observed a clear demarcation between sensitive and resistant cell lines, whereas in bladder cancer, similarly to breast cancer, I observed a wide and fairly continuous range of IC50 values and an almost equal number of cell lines in each group. Among the cell lines belonging to the sensitive group, RT112 stands out for being particularly sensitive to BMS-754807. Interestingly, 4 out of 8 cell lines considered IGF-1R inhibitor sensitive are also known for being FGFR3 dependent (188), suggesting that those cells are dependent on more than one receptor to proliferate. Next, I sought to find out whether the inhibitory effect of BMS-754807 was due to cell cycle arrest or apoptosis. I screened the same cells for apoptosis using cell cycle analysis. Previous studies showed how small molecule inhibitors targeting downstream pathways of the IGF-1 receptor, like the PI3K or the MAPK pathway, mostly showed little or no apoptosis induction in bladder cancer cells, having mainly a cytostatic rather than a cytotoxic effect (164,189,190). I also observed similar results (Fig.3.10B), as none of the 30 cell lines tested showed an increase higher than 10% after baseline subtraction. I also tried to link IGF-1R expression with sensitivity (Fig.3.10C, Fig3.11), but high levels of receptor did not seem to correlate with sensitivity to BMS-754807. To test the on target effects of the IGF-1R inhibitor, I exposed the most sensitive and most resistant cell lines (RT112 and UC16) to increasing concentrations of BMS-754807 under 3 different conditions: 10% FBS media, serum starved and serum starved + soluble human IGF-1 stimulation (Fig. 3.12). As expected, BMS-754807 showed no or very modest effects in UC16 cells on AKT, ERK and S6 phosphorylation. Surprisingly, in RT112, the IGF-1R inhibitor showed consistent dose-dependent effects across the three different conditions only in AKT and S6 phosphorylation but not on ERK phosphorylation, suggesting that its main effects on this specific cell line are mainly affecting the

downstream PI3K pathway but not the MAPK pathway. This observation led us to further investigations, discussed more specifically in the next chapter.

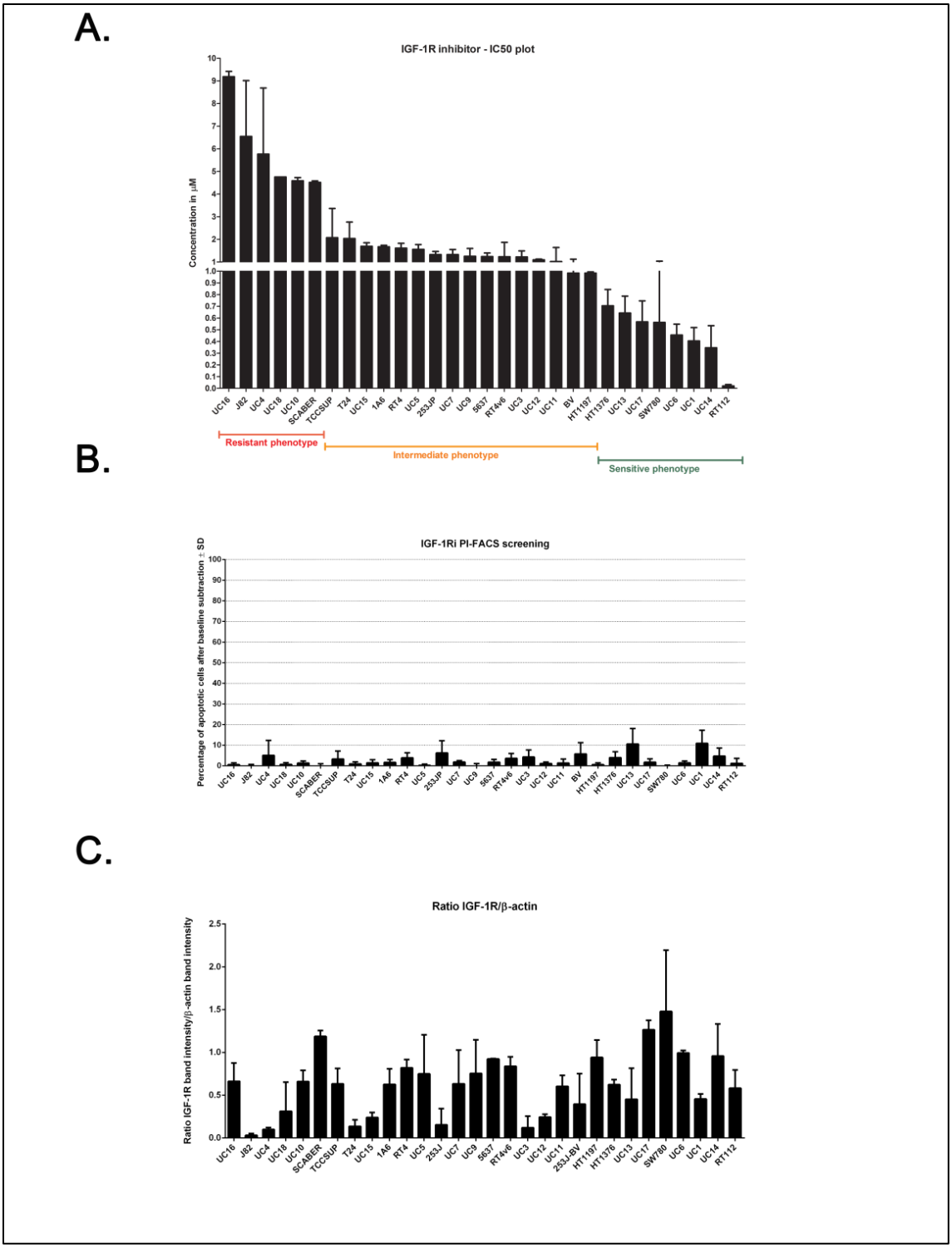


Figure 3.10: Sensitivity of bladder cancer cell lines to increasing concentrations of AZ7328 as measured in a 120 h MTT assay.Relationship between the effects of BMS-754807 on proliferation (A) and apoptosis (B) with basal IGF-1R expression.

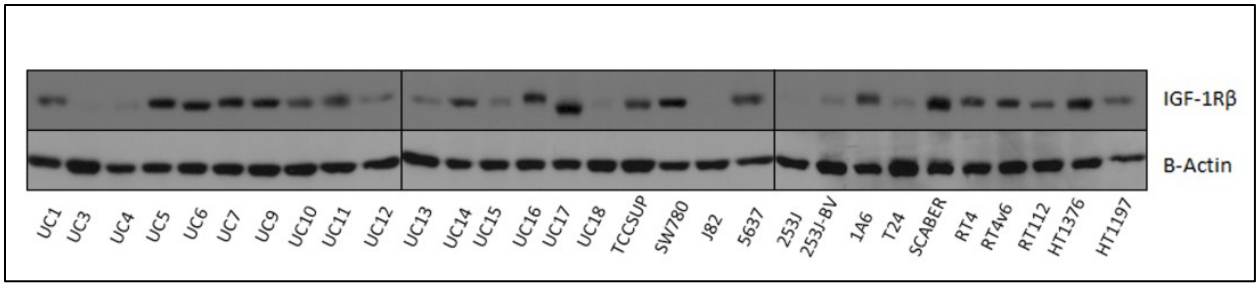
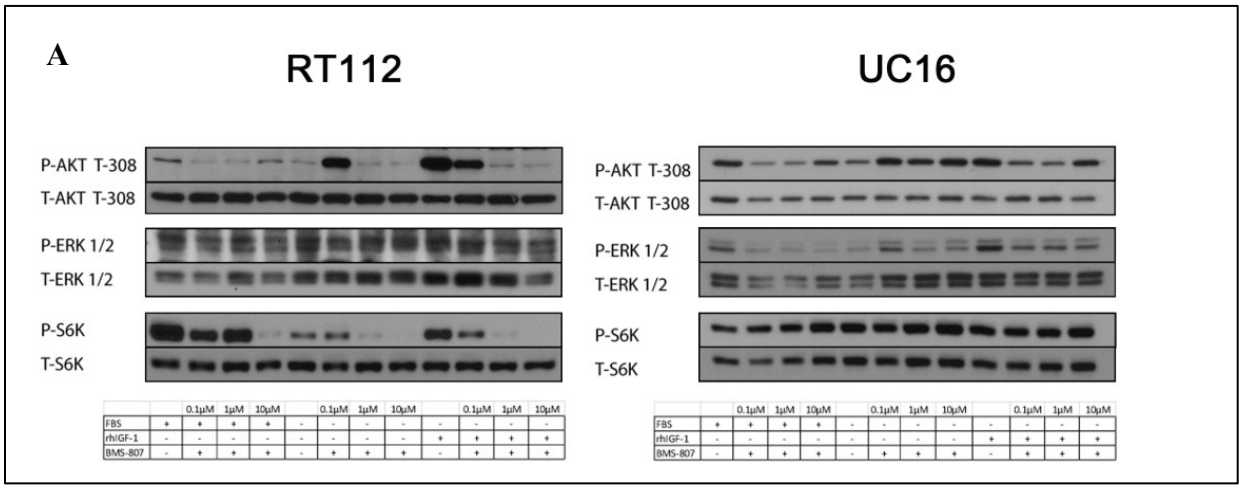


Figure 3.11: Western blots of the basal IGF-1R expression in a panel of 30 bladder cancer cell lines



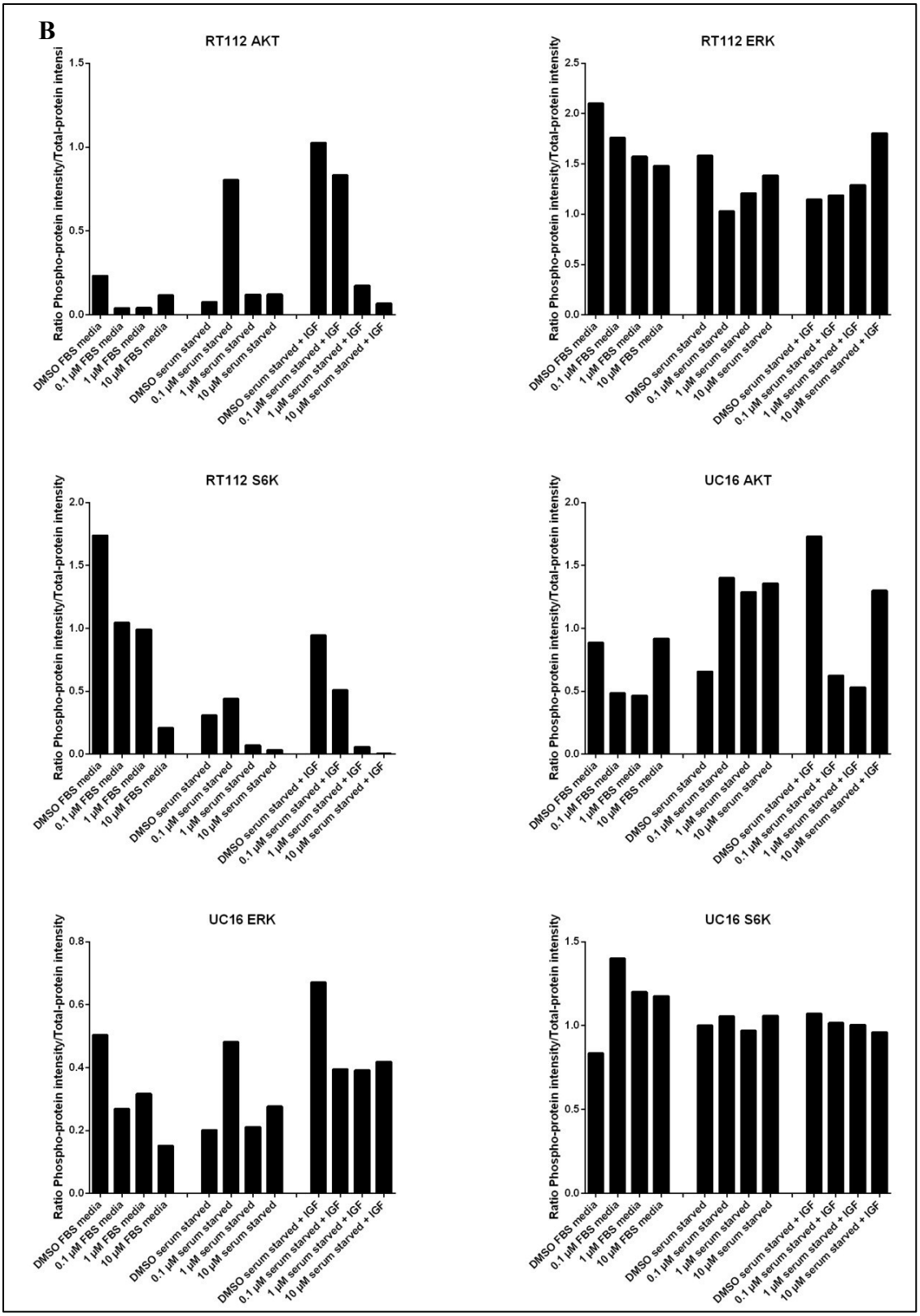


Figure 3.12: Western blots representing the on-target effects of BMS-754807 on downstream targets AKT, ERK1/2, and S6 in the most sensitive and resistant cell lines. The bands were quantified by densitometry and plotted as bar graphs.

CHAPTER 4: INHIBITION OF AUTOPHAGY AS A STRATEGY TO SENSITIZE BLADDER CANCER CELLS TO SMALL MOLECULE INHIBITORS TARGETING THE PI3K PATHWAY

This chapter is partially based upon "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" with the permission of the journal.

4.1.1 Investigation of the effects of the combination of the AKT inhibitor AZ7328 with conventional chemotherapy or other small molecule inhibitors

In a previous study we reported that EGFR inhibition, upstream of PI3K/AKT/mTOR, augmented TRAIL-induced apoptosis via an AKT-dependent mechanism (191). I, therefore, sought to evaluate whether direct AKT inhibition might also enhance apoptosis via the extrinsic pathway. I used PI-FACS to assess apoptosis-associated DNA fragmentation, which emerges as a sub-G1 population upon cell sorting. At 24 h, I observed minimal induction of apoptosis in AZ7328 resistant cell lines (253J B-V), whereas a more sensitive cell line (UM-UC-5), displayed very modest levels of cell death in response to the combination of AZ7328 plus rhTRAIL (Fig. 4.1A and B). Furthermore, neither cell line showed any induction of apoptosis mediated by the intrinsic pathway when exposed to AZ7328 in combination with gemcitabine and/or Cisplatin at clinically relevant concentrations (Fig. 4.1C and D). A subset of these experiments was confirmed by varying cell lines, duration of drug exposure, and drug concentration. Fig 4.2

mTOR is a downstream effector of AKT that is important for many cellular processes, including autophagy, cell cycle progression (from G_0/G_1 to S phase), cell proliferation, angiogenesis and apoptosis. In bladder cancer cell lines, mTOR inhibition with rapamycin has been shown to significantly decrease bladder cancer cell proliferation and induce G_0/G_1 cell-cycle arrest without stimulating apoptosis in vitro and in vivo (189,192,193). In our panel of cell lines, rapamycin induced heterogeneous and partial growth arrest that plateaued at drug concentrations around 10 nM (Fig. 4.3). Interestingly, the patterns of sensitivity to rapamycin and AZ7328 were quite distinct. For example, UM-UC-3 and UM-UC-11, two mesenchymal cell lines were relatively more sensitive to rapamycin than AZ7328, whereas UM-UC-5 and UM-UC-6 were

relatively more sensitive to AZ7328 than rapamycin. Nevertheless, 253J B-V and T24 were highly resistant to both AKT and rapamycin.

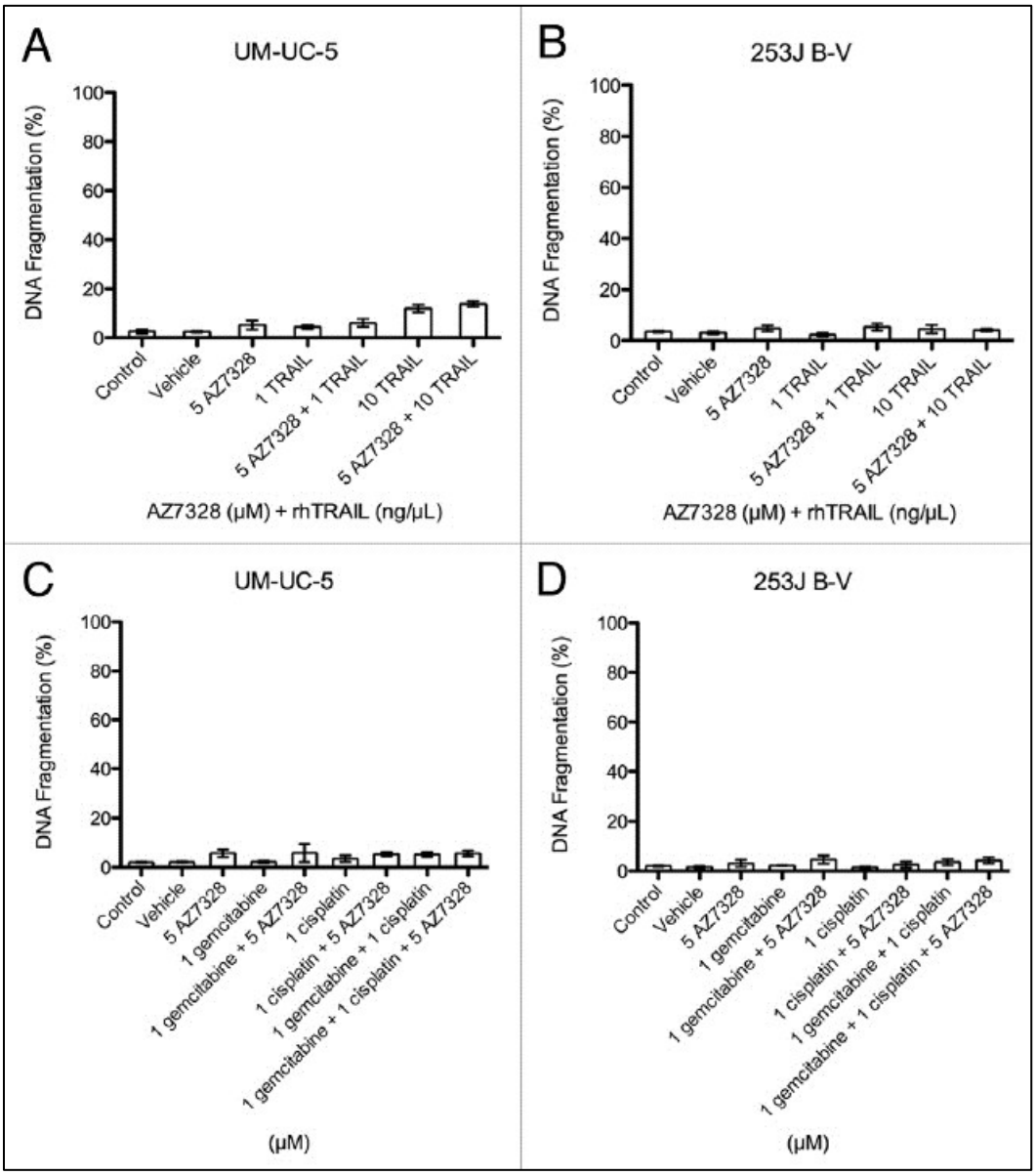


Figure 4. 1: Effects of AZ7328 on apoptosis. Bladder cancer cell lines were exposed to 5 μM AZ7328 alone and in combination with either 1 ng/mL or 10 ng/mL rhTRAIL or chemotherapy. Apoptotic cells were quantified by PI-FACS. (**A**) Effects of AZ7328 with or without TRAIL in a sensitive cell line (UM-UC-5). (**B**) Effects of AZ7328 with or without TRAIL in a resistant cell line (253J B-V). (**C**) Effects of AZ7328 with or without various combinations of both 1 μM gemcitabine and 1 μM cisplatin in a sensitive cell line (UM-UC-5). (**D**) Effects of AZ7328 with or without various combinations of both 1 μM gemcitabine and 1 μM cisplatin in a resistant cell line (253J B-V). Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

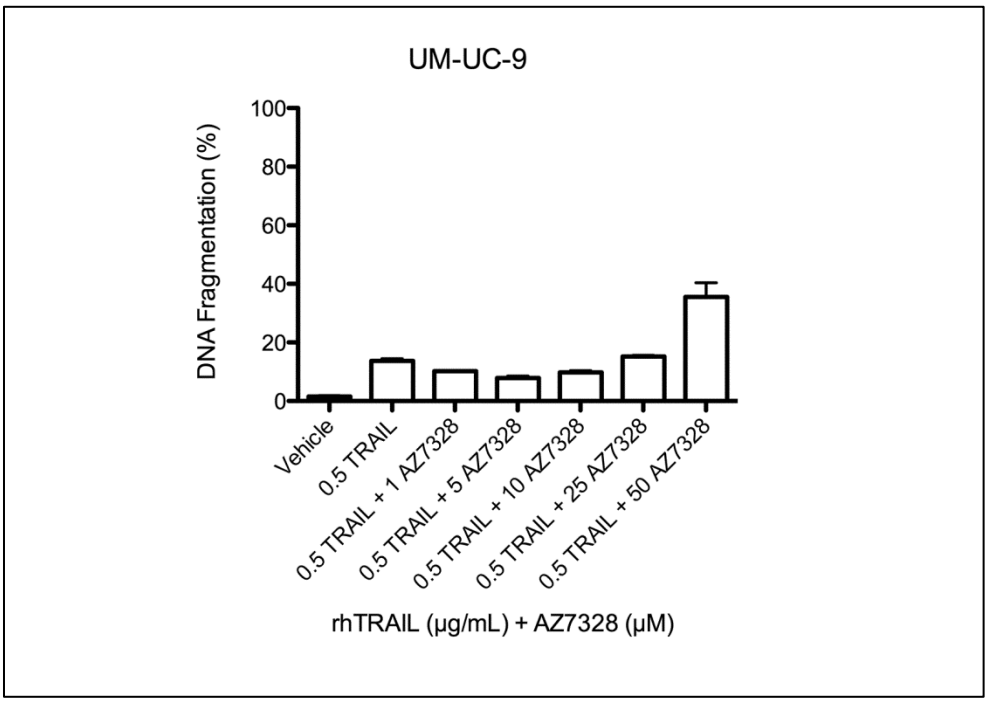
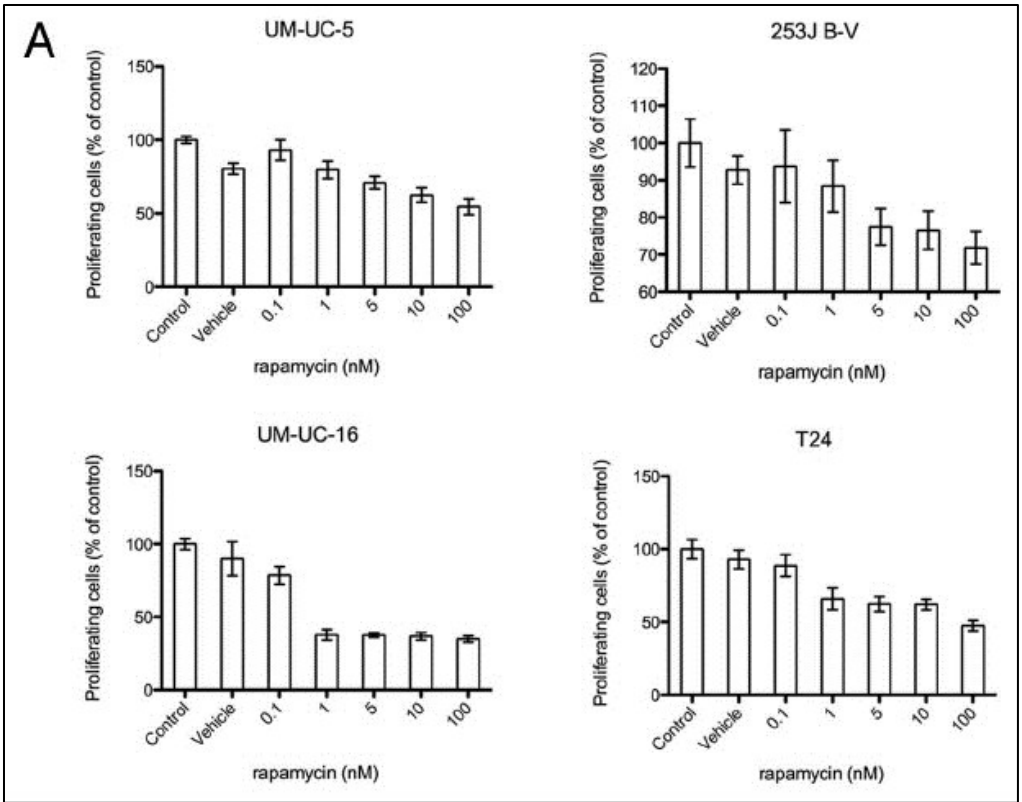


Figure 4.2: Combination of a fixed dose of TRAIL with increasing concentrations of AZ7328 on TRAIL sensitive cell line UM-UC-9. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.



B

	% Growth Inhibition at 5 nm		% Growth Inhibition at 10 nm
UM-UC-16 [b,d]	62.4	UM-UC-16 [b,d]	63.2
UM-UC-10	56.1	UM-UC-3 [d,e]	56.4
UM-UC-3 [d,e]	54.1	UM-UC-10	53.1
UM-UC-11	48.0	J82 [b,d]	49.6
J82 [b,d]	42.9	UM-UC-11	45.9
T24 [c,e]	37.6	UM-UC-6 [b,c,d]	13.6
UM-UC-9	33.7	UM-UC-5 [a,d]	37.6
UM-UC-5 [a,d]	29.0	UM-UC-13	37.2
253J B-V [d]	22.5	UM-UC-14 [b]	34.6
UM-UC-6 [b,c,d]	1.9	T24 [c,e]	33.0
UM-UC-13	-5.3	253J B-V [d]	23.4
UM-UC-14 [b]	-18.7	UM-UC-9	20.5

Sensitivity

Figure 4.3: Sensitivity of bladder cancer cell lines to increasing concentrations of rapamycin as measured in a 120 h MTT assay. (**A**) The anti-proliferative effects of rapamycin in four cell lines (UM-UC-5, UM-UC-16, 253J B-V and T24). Note the differences in scales. (**B**) Rank ordering of sensitivity to rapamycin at 120 h of exposure in a panel of 12 bladder cancer cell lines by the percentage of proliferative inhibition induced at both 5 and 10 μM concentrations. a EGFR amplification, b FGFR3 mutation, c c-MET mutation, d PIK3CA mutation, e RAS mutation. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

It is well known that a feedback downregulation of RTK signaling exists in cells with constitutive mTOR activation. mTOR inhibition relieves this feedback and causes AKT activation, which attenuates the antitumor effects of mTOR inhibitors (194). These preclinical observations have been reproduced in humans (66). Thus, combination therapy targeting both mTOR and AKT may produce enhanced antitumor activity relative to the effects of mTOR inhibition alone. To test this hypothesis, I exposed our panel of bladder cancer cells to the combination of rapamycin plus AZ7328 and I found at least additive effects of the two drugs. This improved response was seen in all but one of the 12 cell lines tested (253J B-V) (Fig. 4.4). Thus, resistance to the cytostatic effects of either single agent alone can be overcome in most bladder cancer cell lines with combination therapy in vitro.

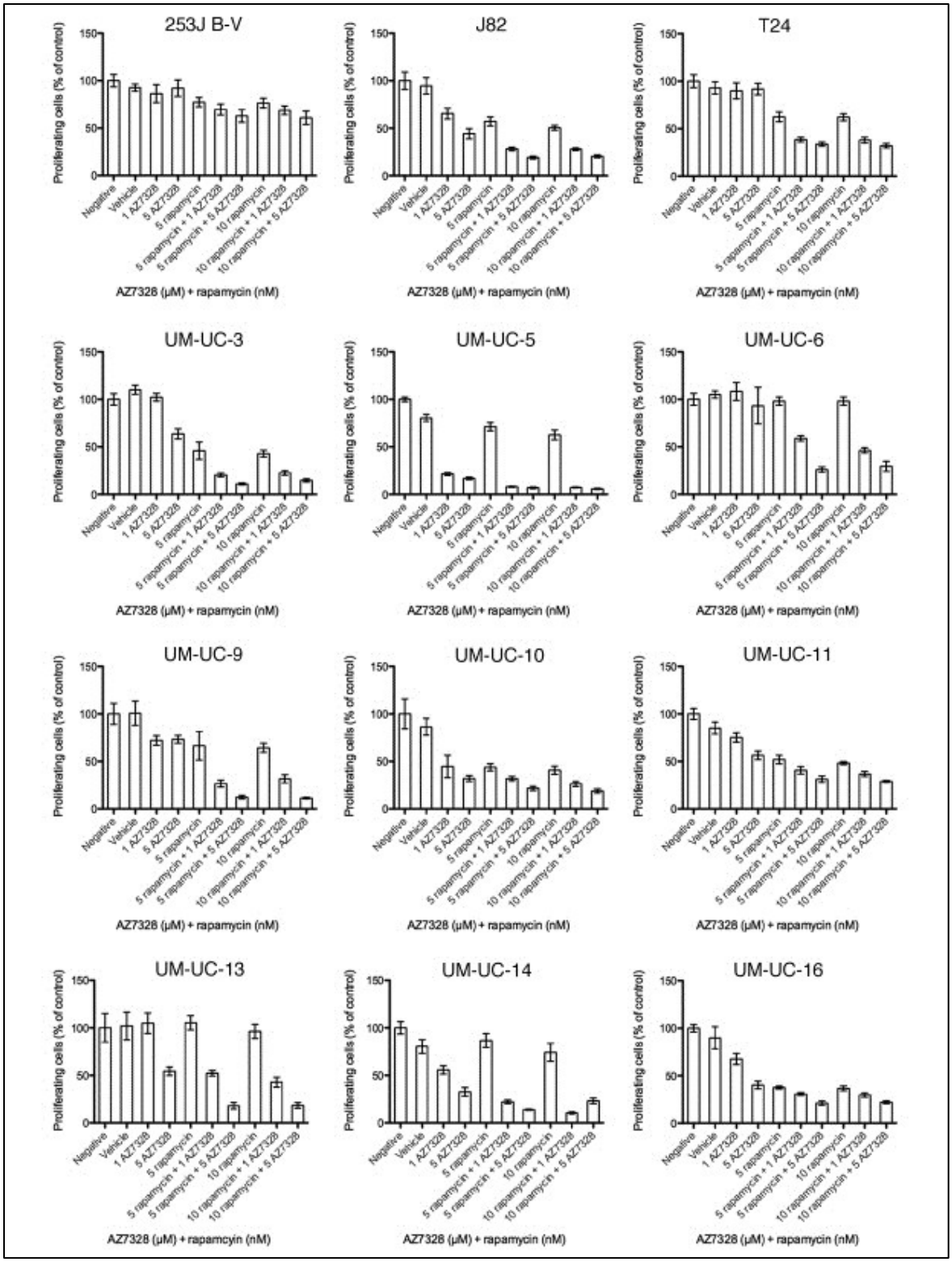


Figure 4.4: Sensitivity of all 12 bladder cancer cell lines to various combinations of AZ7328 and rapamycin as measured in a 120 h MTT assay. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

4.1.2 Block of autophagy coupled with the AKT inhibitor AZ7328 induces apoptosis in some bladder cancer cell lines

Active PI3K/AKT/mTOR signaling is associated with high rates of cellular metabolism and protein synthesis (194). Autophagy is a cytoprotective adaptive response to nutrient deprivation in yeast or the absence of growth factor receptor signaling in metazoans that functions to provide a source of energy and amino acids when extracellular sources are not accessible. One of the major consequences of active mTOR signaling is suppression of autophagy (195,196). I, therefore, questioned whether AZ7328, by virtue of blocking AKT-mediated mTOR signaling, might stimulate autophagy in our cell lines, thereby masking pro-apoptotic effects of AKT inhibition. Consequentially, I hypothesized that activation of autophagy is a cytoprotective mechanism used by bladder cancer cells to evade apoptosis. This idea is corroborated by the fact that as previously mentioned in chapter 1, autophagy has been described like a double-edged sword that has been shown to be utilized by the cells as alternative programmed cell death mechanism, upon extensive and perpetuated cellular stress and starvation, but also used as alternative process to evade apoptosis. To examine this possibility, a select group of cell lines with varying levels of cytostatic response to AKT inhibition were exposed to various concentrations of AZ7328. Autophagy was assessed via anti-LC3 immunoblotting, to identify the accumulation of autophagosome components. AZ7328 induced LC3 processing in three of the four cell lines (UC5, UC9 and J82). We confirmed these results in the J82 and T24 cell lines using anti-LC3 immunofluorescence (197), which clearly revealed drug-induced LC3 punctae, indicating autophagosome formation in the former but not the latter (Fig. 4.5). To determine whether this induction of autophagy was an important cytoprotective mechanism, we exposed the cell lines to AZ7328 plus the chemical autophagy inhibitor Chloroquine and quantified levels

of apoptosis by PI-FACS. The combination of AZ7328 and Chloroquine induced apoptosis in the cells that displayed drug-induced autophagy (UC5, UC9 and J82), but not in the T24 cells (Fig. 4.6). Despite the increase in apoptosis, the levels of cell death achieved were not significantly higher than contols when looking at Chloroquine in combination with 1μM of AKT inhibitor (a realistically clinically relevant concentration). Only in UC5, I could achieve ~ 15% of apoptosis induction, while no difference between controls and drug combinations was observed in UC9 and J82. A possible explanation could be found in the low efficacy of AZ7328, considered to be a first generation AKT inhibitor and probably less potent than other currently available AKT inhibitors. Despite this last observation, my results may still provide a strong rationale for evaluating the toxicity and efficacy of therapies based on improved AKT inhibitors in combination with more specific autophagy inhibitors in preclinical in vivo models and subsequently in bladder cancer patients.

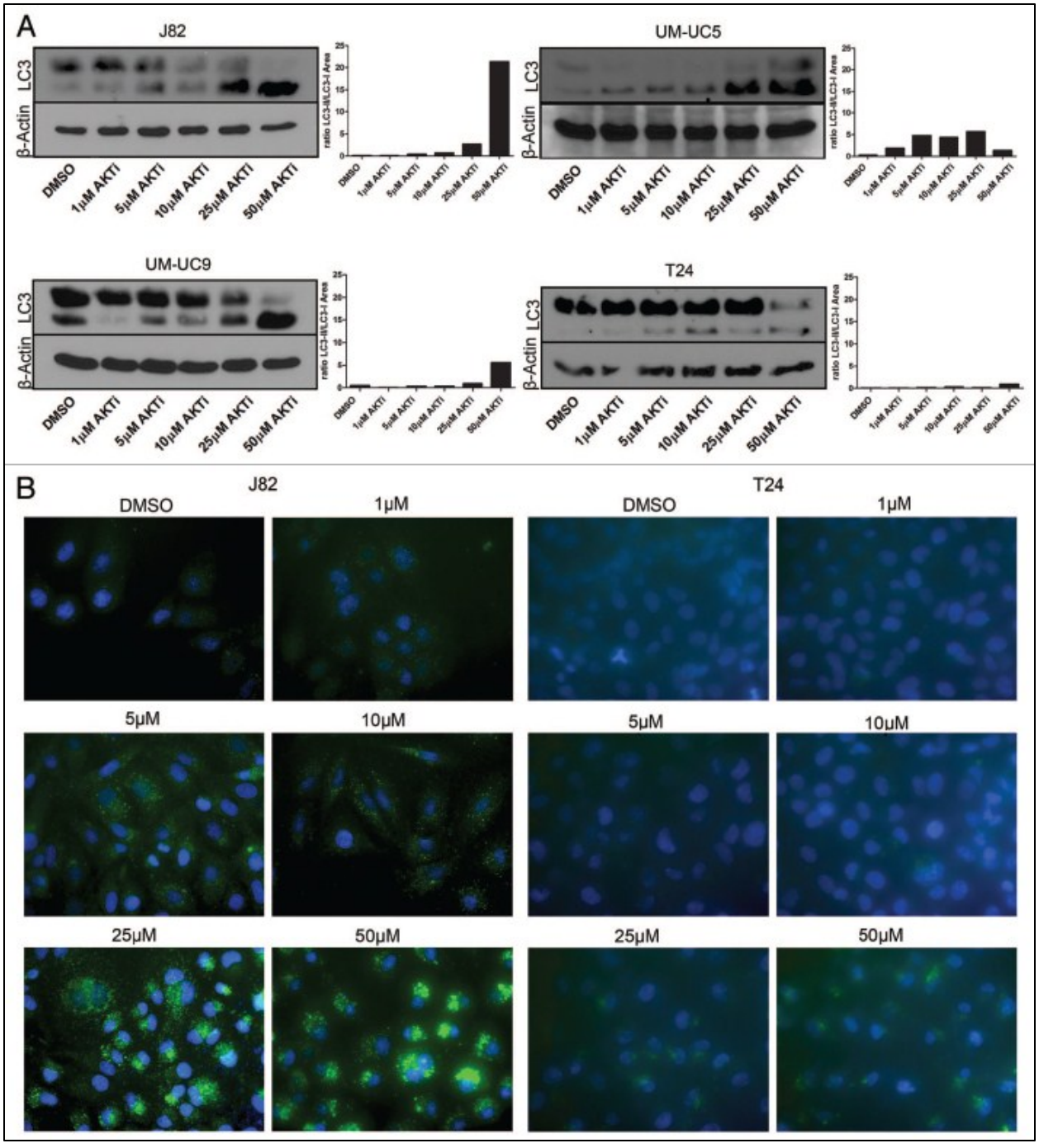


Figure 4.5: Concentration-dependent effects of AZ7328 on autophagy. (A) Immunoblot displaying LC3-I and LC3-II expression in four representative cell lines (J82, UM-UC-5, UM-UC-9 and T24). The LC3 bands were quantified using Image J software and the bar graphs show the ratio of LC3-II to LC3-I as a function of autophagy. (B) Immunofluorescence analysis of LC-3 localization in J82 and T24 cells. Note that punctate LC-3 staining (green) is characteristic of autophagy. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

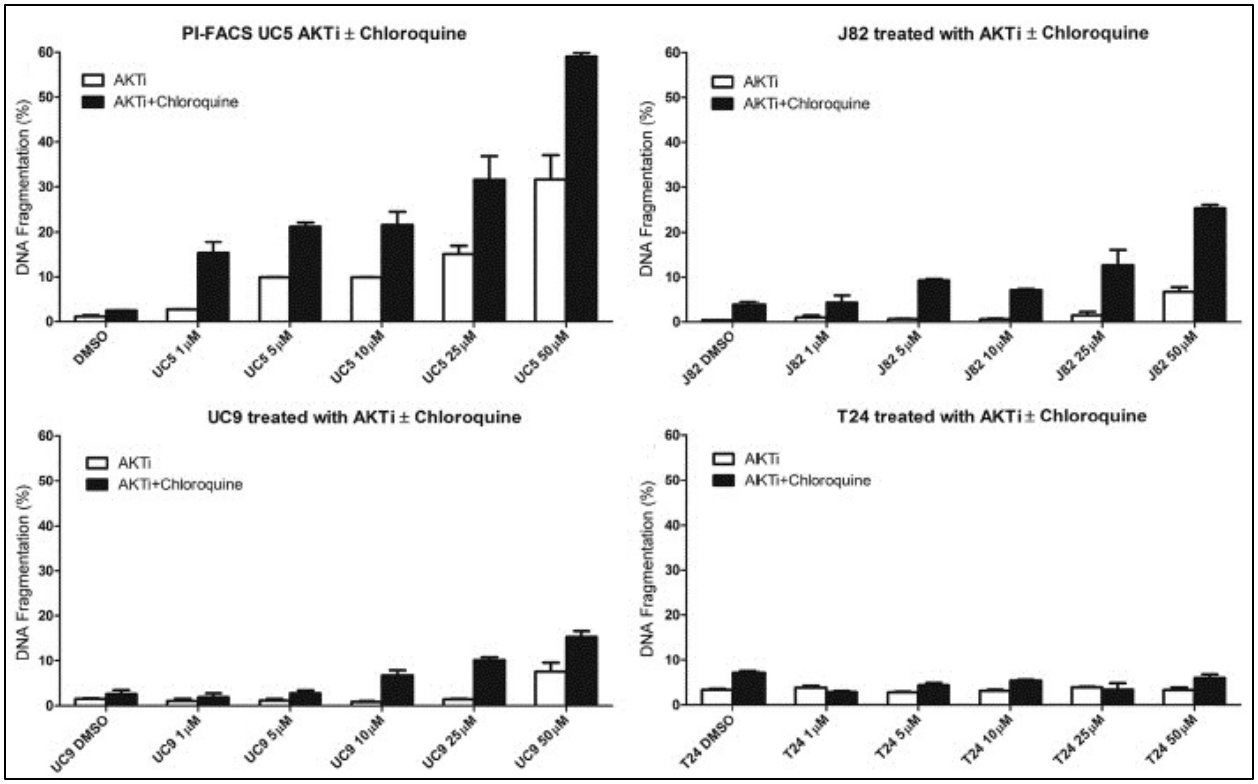


Figure 4.6: Effect of AZ7328 on apoptosis, as single agent or in combination with chloroquine. Bladder cancer cells (J82, UM-UC-5, UM-UC-9 and T24) were exposed to increasing concentrations of AZ7328 alone or in combination with a fixed dose of 50 μM chloroquine. Apoptotic cells were quantified by PI-FACS. Adapted from "Dickstein, R. J., Nitti, G., Dinney, C. P., Davies, B. R., Kamat, A. M., and McConkey, D. J. (2012) Autophagy limits the cytotoxic effects of the AKT inhibitor AZ7328 in human bladder cancer cells. *Cancer Biol Ther* **13**, 1325-1338" (164) with the permission of the journal.

4.1.3 Investigation of the effects of the combination of the mTOR inhibitor AZ4274 with conventional chemotherapy or other small molecule inhibitors

A large subset of bladder cancer cell lines show mutations in several components of the PI3K pathway (11) and mTOR, in particular, has been shown to play an important role in cell growth (198). Currently available inhibitors, such as RAD001 and rapamycin, only target mTORC1 showing a wide spectrum of inhibition on different cell lines. Our novel mTOR inhibitor targets both mTORC1 and mTORC2. Our initial hypothesis was that by being able to target the same pathway twice, I would observe better growth inhibition in comparison to drugs designed to hit only a single component of the PI3K pathway. In order to test our hypothesis, I first screened a

panel of 11 cell lines with our mTOR inhibitor to estimate the IC50 of our drug. All of the cell lines tested by MTT assay were inhibited by AZD-4264 in a dose-dependent fashion (Figure 4.7A and 4.7B). Surprisingly, all the cell lines tested showed a sensitive trend. The IC50 was achieved at sub-micromolar levels for 7 out of 11 cell lines tested (Figure 4.7C). Even UC9, our most resistant cell line, showed an IC50 between 2 and 3 μM, which is not much higher than our most sensitive cell line, UC13. Interestingly, the plot of sensitivity for AZD-4264 and the AKT inhibitor AZ7328 seemed to be consistent among the same cell lines. Both the inhibitors include UC5 and UC14 among the most sensitive cells and T24, UC9 and 253J-BV among the most resistant cell lines. This result was expected, given the fact that both inhibitors target the same cellular pathway and are therefore likely to be influenced by the same molecular alterations. Major outliers are UC3 and UC13, notably, both mesenchymal cell lines. Similarly to what previously discussed the pattern of sensitivity for AZD-4264 is surprisingly different from the one observed with rapamycin, despite the fact that both the inhibitors target TORC1 one. UC14 and UC13 for example are among the most sensitive cells to AZD-4264 while figuring as the most resistant cell lines to rapamycin. It is tempting to speculate that the fact that the AKT and the double mTOR inhibitors share similar pattern of sensitivity, while the effects of rapamycin are so inconsistent, may be due to the fact that both AZD-4264 and AZ7328 directly affect AKT along with all its downstream target, while rapamycin only affects TORC1.Conversely, the waterfall plot of sensitivity for BMS754807 resulted to be different form the ones observed with the other two drugs. This could be due to the fact that IGF-1R regulates several different cell pathways simultaneously, while AZD-4264 and AZ7328 only focused on the PI3K/AKT pathway. It is reasonable to speculate that possible rebound effects may be triggered by the block of this pathway, like, for instance, the activation of the MAPK pathway. In this case, cells

exposed to the IGF-1R inhibitor would show a partially attenuated rebound effect compared to those exposed to the AKT and mTOR inhibitors.

Next, I sought to confirm the bi-dimensional proliferation data obtained by MTT using soft agar culture as a closer *in vitro* model to real tumors. This assay performed on UC9 and UC13 cells confirmed a dose-dependent growth inhibition of both our cell lines (Figure 4.8A), demonstrating how UC9 is more resistant to the drug compared to UC13 (Figure 4.8A and 4.8B). I then compared the percent of growth inhibition in both assays, observing high reproducibility between both assays (Figure 4.8C).

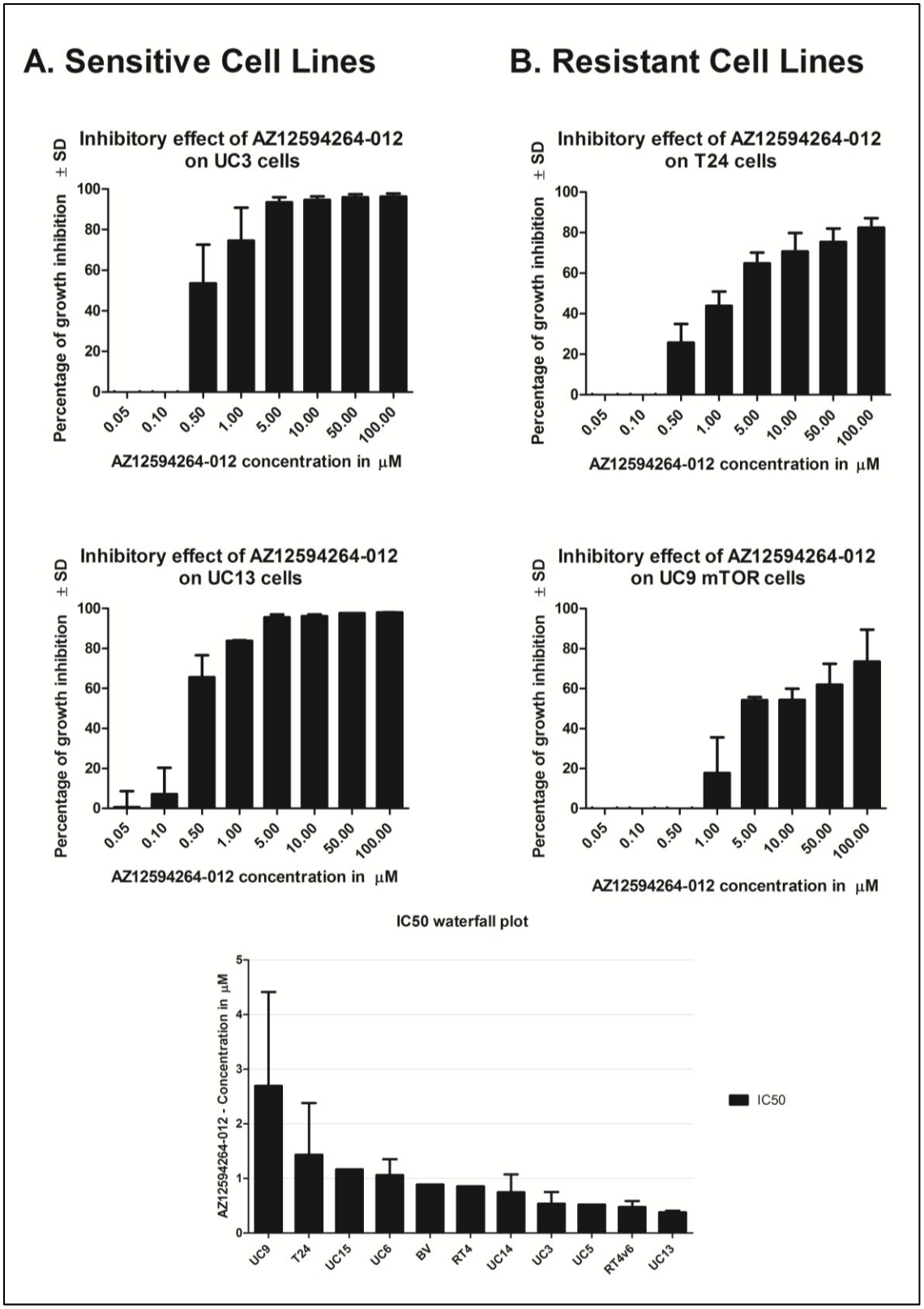


Figure 4.7: Description of the dose-dependent anti-proliferative effects of AZ4264 shown in detail on 2 sensitive and 2 resistant cell lines in A and B and as IC50 on a panel of 11 bladder cancer cell lines in C.

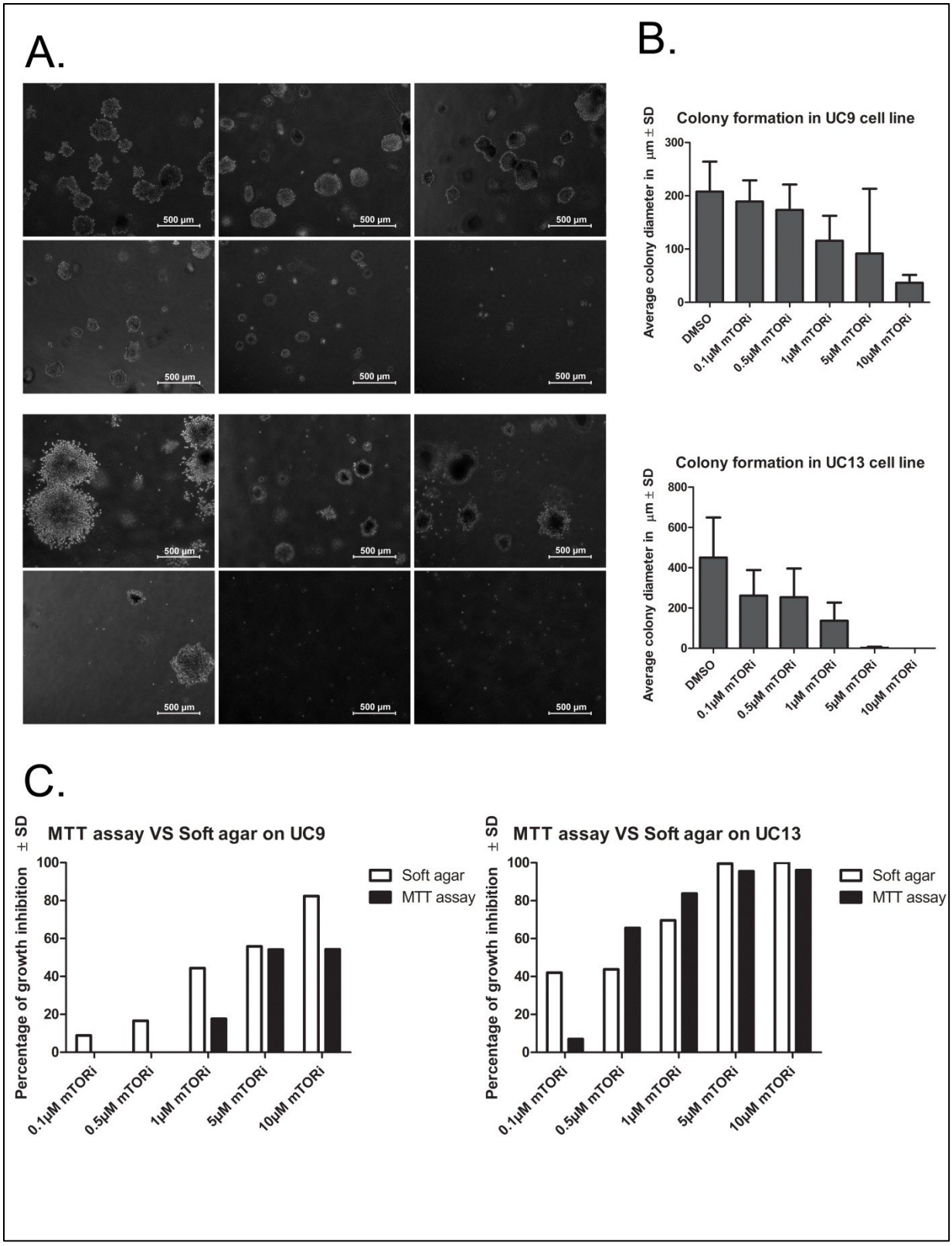


Figure 4.8: Dose-dependent anti proliferative effects of AZ4264 on soft agar colony formation in a sensitive and resistant cell line (A and B). The results were then normalized as percentage of inhibition and compared with data derived from the MTT assays(C)

I then exposed 5 cell lines (UC13, UC3, UC14, T24 and UC9) to increasing concentrations of AZD-4264 and checked the phosphorylation status of its downstream targets by western blot. Phosphorylated downstream targets of mTOR, AKT (Ser473), 4EBP1 and p70-S6K, were all generally inhibited in a dose dependent fashion (Figure 4.9). Interestingly, some residual phosphorylation was observed for some targets (4EBP1 in UC9 and UC3 cells and p70-S6K in UC13), while no effects on the phosphorylation of p70-S6K were observed in T24 cells.

Because the main issue with small molecule inhibitors targeting the PI3K pathway in bladder cancer is represented by its lack of cytotoxicity (164,189), I tested the ability of AZD-4264 to induce DNA fragmentation alone or in combination with conventional chemotherapeutic drugs like Gemcitabine/Cisplatin and TRAIL by PI-FACS (Figure 4.10). In none of the 6 cell lines tested did the mTOR inhibitor cause any cytotoxic effect. When combined with conventional chemotherapeutic drugs, neither synergistic nor additive effects were observed. The cell death detected in UC3, UC6, UC13, UC14 and T24 was entirely due to either a combination of Gemcitabine and Cisplatin or to the effects of TRAIL.

I next sought to improve the growth inhibitory effects of small molecule inhibitors in bladder cancer by targeting the PI3K and MAPK pathways simultaneously. A MEK inhibitor (ADZ-6244) was combined with the double mTOR inhibitor AZD-4264 and AKT inhibitor AZD-7328 (Figure 4.11). The combination of MEK inhibitor with AKT or mTOR inhibitor seemed to be beneficial only in resistant cell lines, like UC9 and T24, with no appreciable results observed on sensitive cell lines, like UC13 and UC3, which showed sub-micromolar IC50s for AZD-4264.

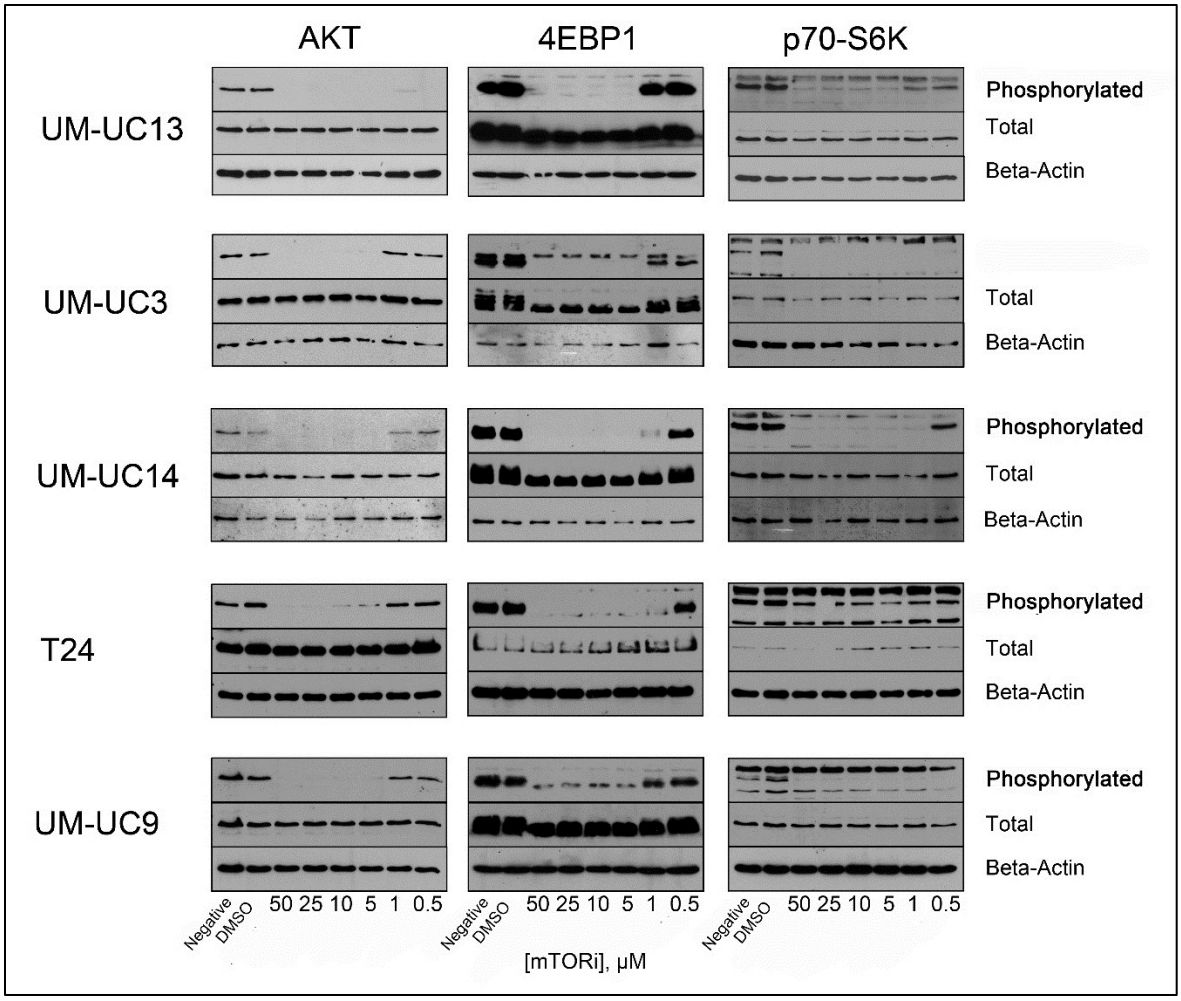


Figure 4.9: Western blots representing the on-target effects of the mTORi AZ4264 on its downstream targets in resistant and sensitive cell lines.

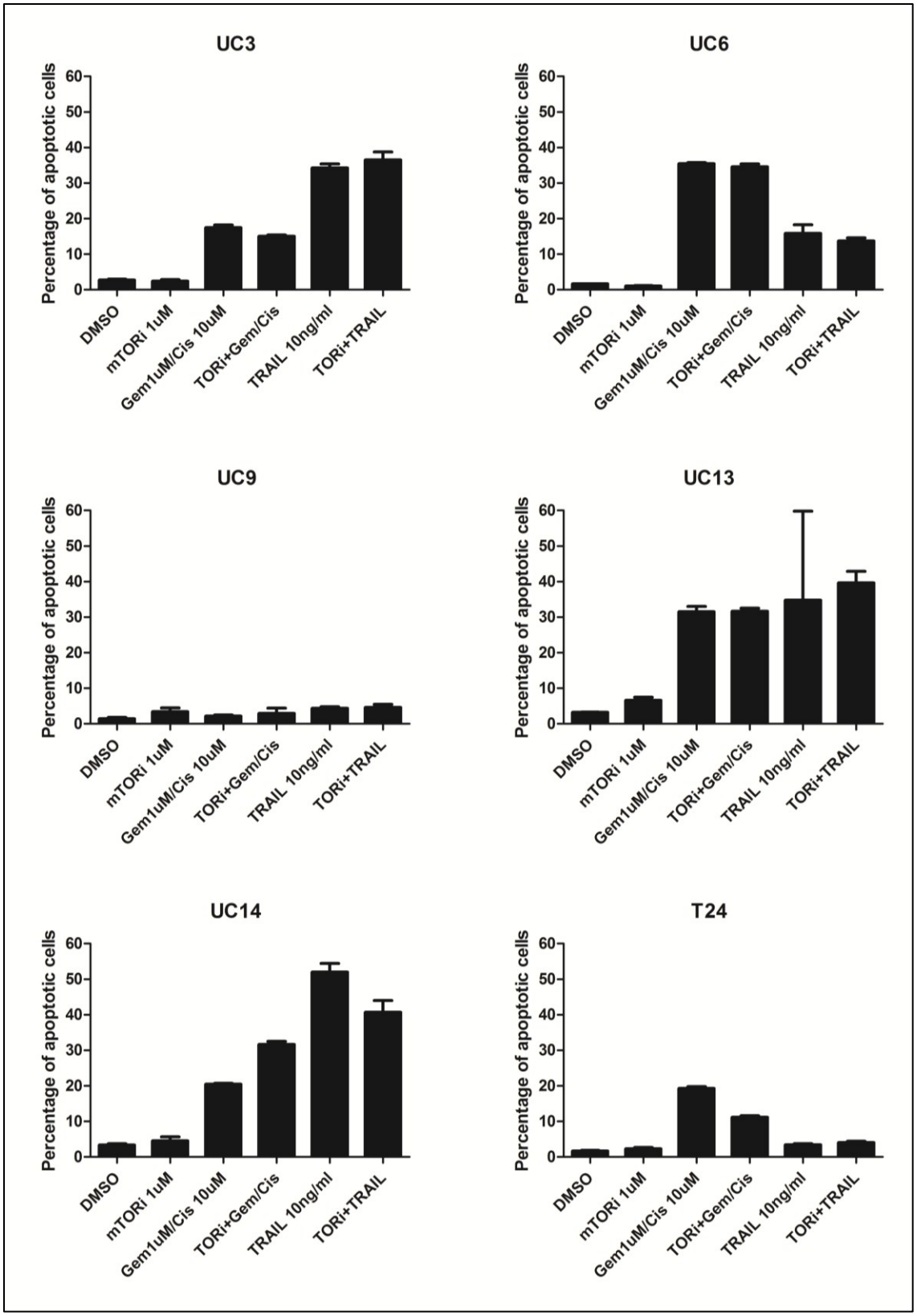
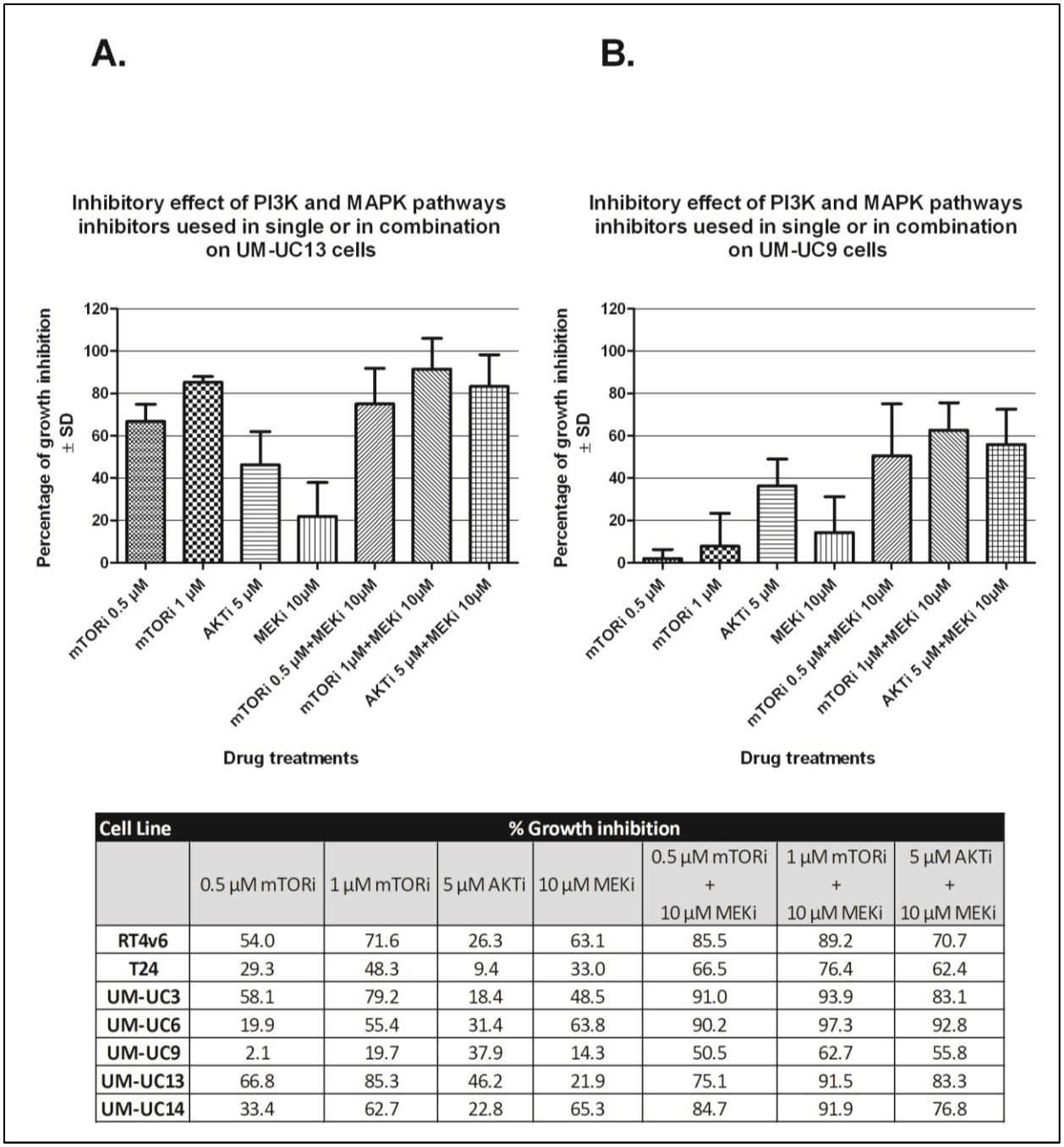


Figure 4.10. Effects on apoptosis from the mTOR inhibitor AZ4264 alone and in combination with convention chemotherapeutic drugs TRAIL and Gem/Cis on 6 cell lines.



Cell Line	% Growth inhibition						
	0.5 µM mTORi	1 µM mTORi	5 µM AKTi	10 µM MEKi	0.5 µM mTORi + 10 µM MEKi	1 µM mTORi + 10 µM MEKi	5 µM AKTi + 10 µM MEKi
RT4v6	54.0	71.6	26.3	63.1	85.5	89.2	70.7
T24	29.3	48.3	9.4	33.0	66.5	76.4	62.4
UM-UC3	58.1	79.2	18.4	48.5	91.0	93.9	83.1
UM-UC6	19.9	55.4	31.4	63.8	90.2	97.3	92.8
UM-UC9	2.1	19.7	37.9	14.3	50.5	62.7	55.8
UM-UC13	66.8	85.3	46.2	21.9	75.1	91.5	83.3
UM-UC14	33.4	62.7	22.8	65.3	84.7	91.9	76.8

Figure 4.11. Effects on cell proliferation from the combination of the mTOR inhibitor AZ4264 with other small molecule inhibitors (AKT and MEK inhibitors) shown in detail in a sensitive and a resistant cell line (A and B) and on a panel of 7 bladder cancer cell lines (C)

4.1.4 Block of autophagy coupled with the mTOR inhibitor AZ4264 induces apoptosis in some bladder cancer cell lines

The encouraging results achieved with the AKT inhibitor AZD-7328, made us wonder whether the autophagy activation observed when bladder cancer cells were exposed to the AKT inhibitor due to an off target effect of the drug or a phenomenon derived by the inhibition of the downstream targets of the PI3K pathway. To address these possibilities I exposed 4 cell lines (UC3, UC13, UC14 and T24) to increasing doses of AZD-4264 and detected LC3-I and LC-3-II expression by western blot (Figure 4.12 top). I estimated activation of autophagy by the ratio between LC3-II and LC3-I bands, as previously published (199). In all but one (T24 cells) of the analyzed cell lines, AZD-4264 was able to activate autophagy in a dose dependent manner. I then confirmed this finding by LC-3 punctae immunofluorescence in a cell line that activated autophagy upon exposure to the mTOR inhibitor (UC14) and in a cell line that did not activate autophagy (T24) (Figure 4.12 bottom).

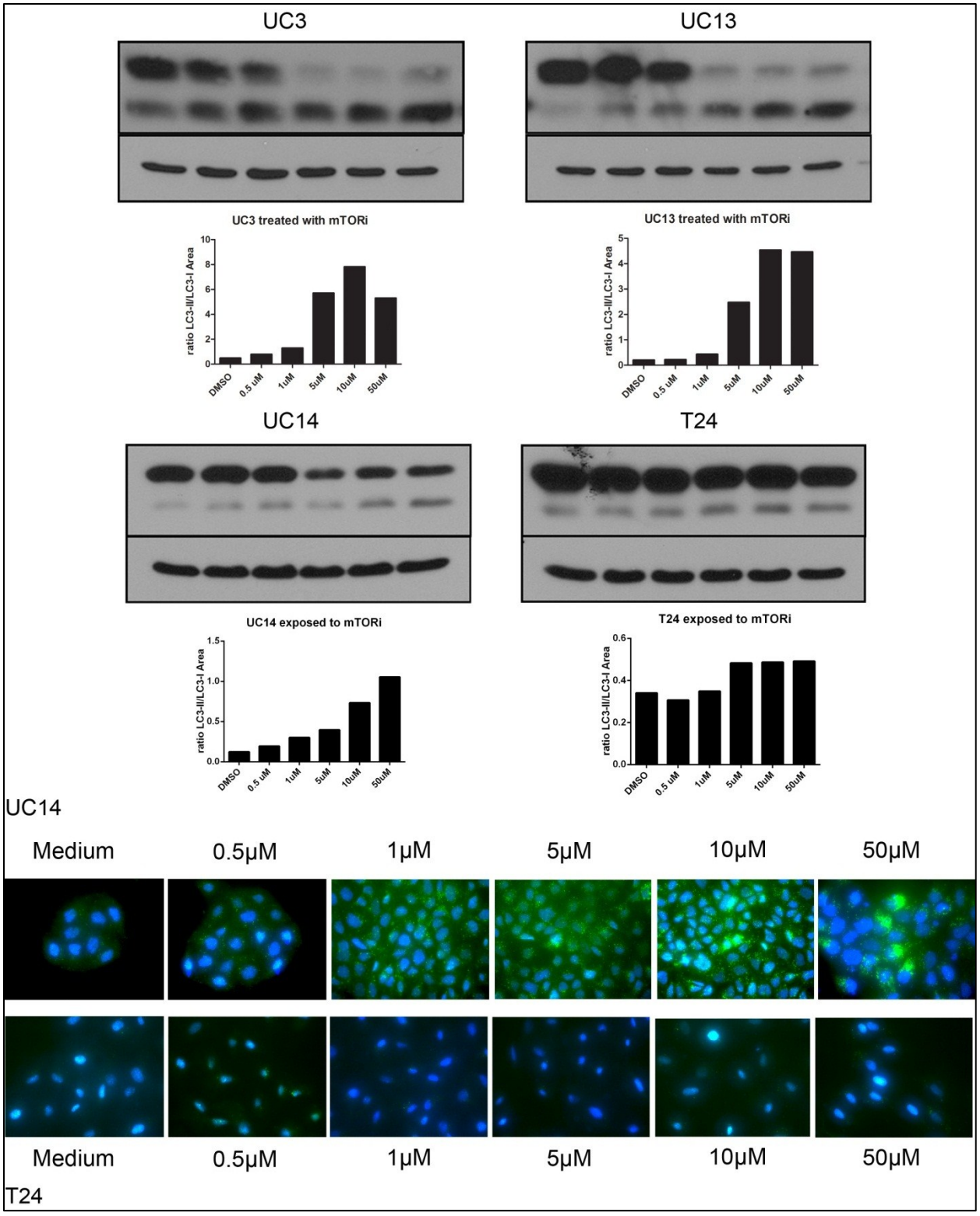


Figure 4.12: Dose dependent effects of AZ4264 on autophagy activation measured as LCII/LC3I ratio by western blot and confirmed by LC3 punctae immunofluorescence

In the beginning of this project, I expected AZD-4264 to be cytotoxic for bladder cancer cells because the PI3K/AKT pathway regulates pro-survival pathways. I then hypothesized, similar to what was observed with AZ7328, that autophagy would represent a cytoprotective mechanism in bladder cancer cells. To validate this hypothesis I blocked autophagy by pre-exposing cells to the chemical autophagy inhibitor Chloroquine and then exposing the cells to increasing doses of AZD-4264 (Figure 4.13A). This combination of drugs showed significant increase in the level of DNA fragmentation in UC13 and UC14 cells (~50-60% of cell death), but not in UC3 and T24 cells, confirming the crucial cytoprotective role of autophagy in bladder cancer.

Because of these encouraging results, I decided to validate the *in vitro* effects of this combination in xenograft models. To do so, I had to switch from AZD-4264 to its clinical counterpart, AZD-8055. As a first step I compared the key effects of the two drugs by MTT to confirm its potent anti-proliferative potential (Figure 4.14). In our hands, AZD8055 was even more potent than AZD-4264 (7-14 folds) and was able to activate autophagy in UC14 cells, but not in T24 cells, just like AZD-4264. Comparing AZD8055's IC50s with previously published work in other types of cancer, I found our results to be very consistent (168). More importantly, the combination of AZD-8055 with Chloroquine showed comparable cytotoxic effects (Figure 4.13B). I also confirmed this last experiment by genetically blocking autophagy, knocking down two key regulatory genes as ATG5 and ATG7 in two cell lines (UC14 and T24), and exposing them to 1 and 5 μM of AZD8055 (Figure15). The level of knock down has been estimated by RT-PCR and western blot. The results confirmed that autophagy inhibition coupled with mTOR inhibition promotes cell death by apoptosis in the cell lines that activate autophagy in response to TOR inhibition. Compared with the data previously shown with the AKT inhibitor, the level of

apoptosis achieved combining 1μM of mTOR inhibitor and Chloroquine seemed to be greatly improved. To a modest 15% of apoptosis in UC5 with the AZ7328, I was able to achieve 25% to 50% of apoptosis respectively in UC14 and UC13 by using AZD8055. These data support the value of future studies to better investigate the potential benefits of targeted therapies coupled with more effective autophagy inhibitors.

On the other hand, one of the most challenging tasks in cancer research is to prevent relapses in patients. A level of apoptosis between 25 and 50% is likely to provide partial tumor shrinkage in the beginning of the treatment but it would also be expected to escape the selective pressure and to keep on growing. One of the possible reasons why not all the cells die when exposed to my combination could be linked to relief of feedback, leading to the activation of compensatory pathways or alternatively the possible mutilation or silencing of key genes in the PI3K pathway.

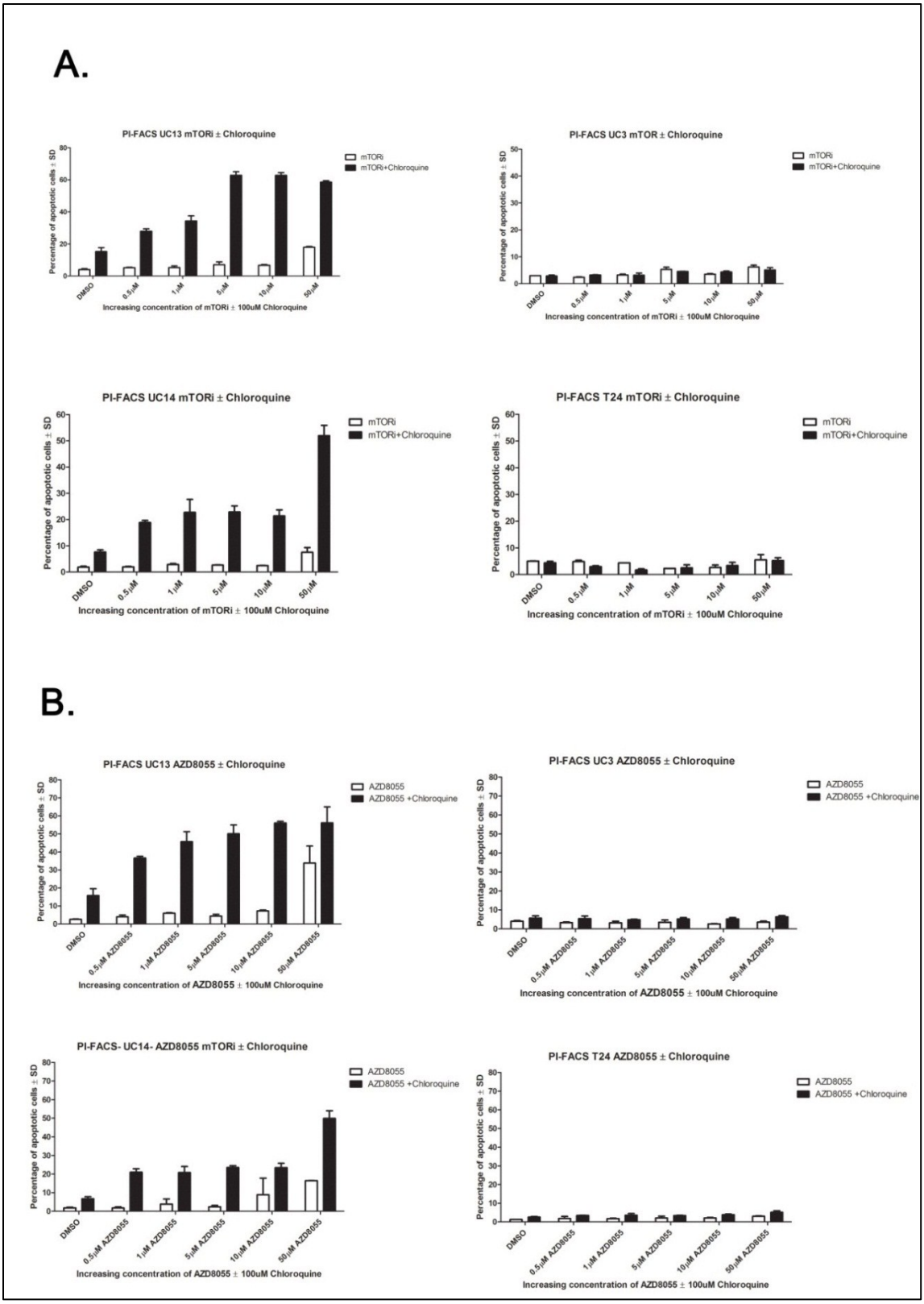
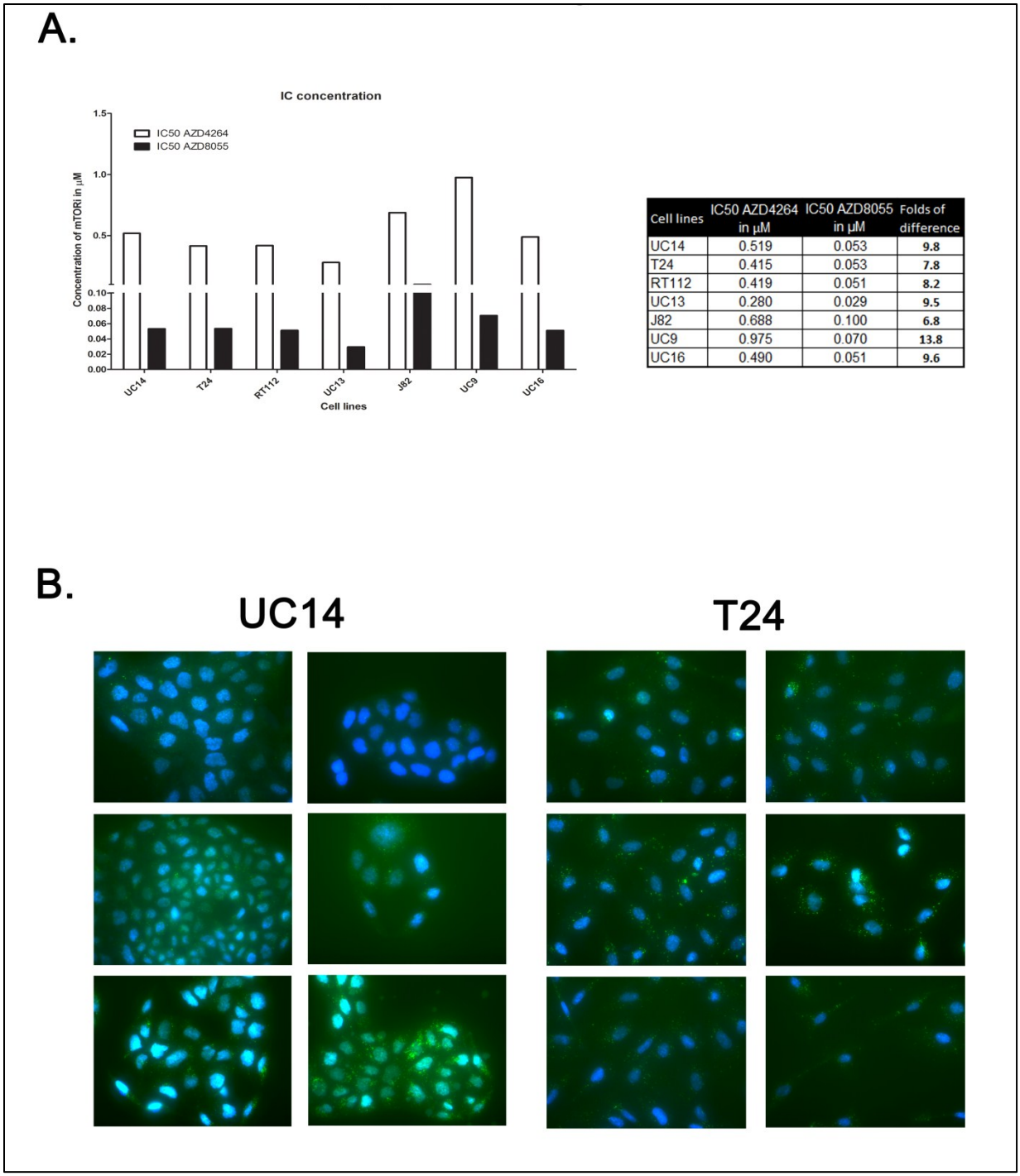


Figure 4.13: Effects of AZD 4264 (A) and AZD8055 (B) on apoptosis when combined with the chemical inhibitor of autophagy Chloroquine in two autophagy activating cell lines (UC14 and UC13) and in two cell lines that did not activate autophagy when exposed to mTOR inhibitor (T24 and UC3).



Cell lines	IC50 AZD4264 in µM	IC50 AZD8055 in µM	Folds of difference
UC14	0.519	0.053	**9.8**
T24	0.415	0.053	**7.8**
RT112	0.419	0.051	**8.2**
UC13	0.280	0.029	**9.5**
J82	0.688	0.100	**6.8**
UC9	0.975	0.070	**13.8**
UC16	0.490	0.051	**9.6**

Figure 4.14: Comparison between the anti-proliferative effects of AZ4264 and its clinical counterpart AZD8055 expressed in IC50 values (A). AZD8055 induces autophagy activation in UC14 but not in T24 just like AZ4264 by LC3 punctae immunofluorescence (B)

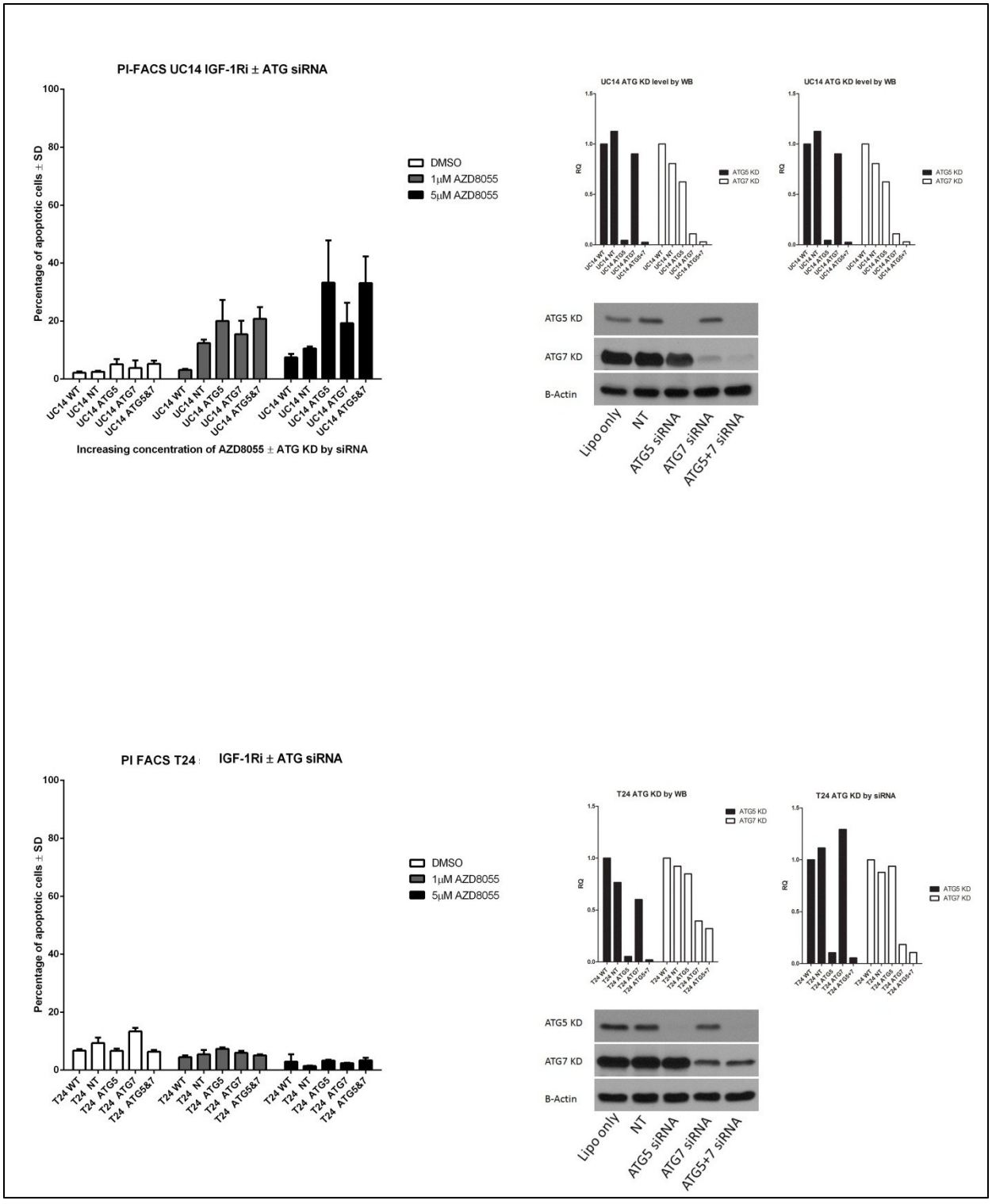


Figure 4.15: Effects of AZD8055 on ATG5 and ATG7 knock down cells UC14 and T24. UC14 is an activator of autophagy when normally exposed to AZ8055 while T24 did not show any autophagy activation

4.1.5 Combination of the mTOR inhibitor AZD8055 and Chloroquine induces apoptosis in mouse models

Next, I sought to verify the effects of the combination of Chloroquine and AZD8055 observed *in vitro* in a 4 arms xenograft study. I orthotopically implanted 120.000 cells in the bladder wall of nude mice and randomized them into 4 groups: a control group that received no treatment, a Chloroquine only group that received 50mg/Kg via IP injection 3 times/week, an AZD-8055 group that received 20mg/kg of drug daily by oral gavage and a combination (combo) group that received both drugs. I began the treatment 10 days after tumor implantation and monitored tumor growth by photon count imaging techniques. At day 28 post treatment, I could notice a statistically significant difference between Control VS Combo, AZD8055 VS Chloroquine, and Chloroquine VS Combo but not between AZD8055 VS Combo, Control VS Chloroquine and AZD8055 VS Combo (Figure 4.16). It is interesting that the combo group showed a marked regression in tumor size after 14 days of treatment, perhaps due to induction of apoptosis. Unfortunately, despite the drop in photon count still no significant difference was observed between AZD-8055 and the combination group. Unexpectedly, the control group also seemed to have a slight drop in photon count at day 14 that was not consistent on day 21 and 28, suggesting that the tumor growth in control group plateaued after 2-3 weeks. The bladder weight analysis in figure 4.17 shows how there is a real difference between the AZD-8055 group and the control group partially disproving what observe in figure 4.16. The statistical analysis derived from bladder weights reflects better our initial expectations about the study: showing a clear anti -tumor effect in the groups treated with AZD-8055 and the combination of AZD-8055 and CQ versus the control and Chloroquine groups. Unfortunately no significant difference was observed between the AZD-8055 and combo group, even though the p-value was close to be significant

(0.069). One likely explanation is that we took in account the weight of the whole bladder of each mouse instead of the actual tumor weight. In conditions where bladder weight is measured in centigrams, considerable variations of for example 3-5 folds in small tumors that weight in the range micrograms are likely to be underestimated.

The next step was to histologically analyze the effects of each different condition on tumors, by staining them with hematoxylin and eosin, Ki67 and Caspase 3 to test their cytostatic and cytotoxic potential (Figure 4.18). Surprisingly, no difference was noticed in Ki67 staining across each group and no apoptosis was observed in any of the analyzed specimens. Ki67 is a marker of proliferation that stains cells in G1, M, S or G2 phase but not in G0. A possible explanation for the lack of difference across each different group could be that AZD-8055 blocks cells in G1 rather than in G0, also staining cells that are not actively proliferating. To test this hypothesis, I am planning to repeat the staining with a better marker of proliferation like for example Cyclin D1 that would stain cells at later stages. Finally, I believe that Caspase 3 staining maybe underestimate the level of apoptosis in my cells because of the low specificity of the antibody used for this immunohistochemistry exam. I am thus planning to test induction of apoptosis by TUNEL assay instead.

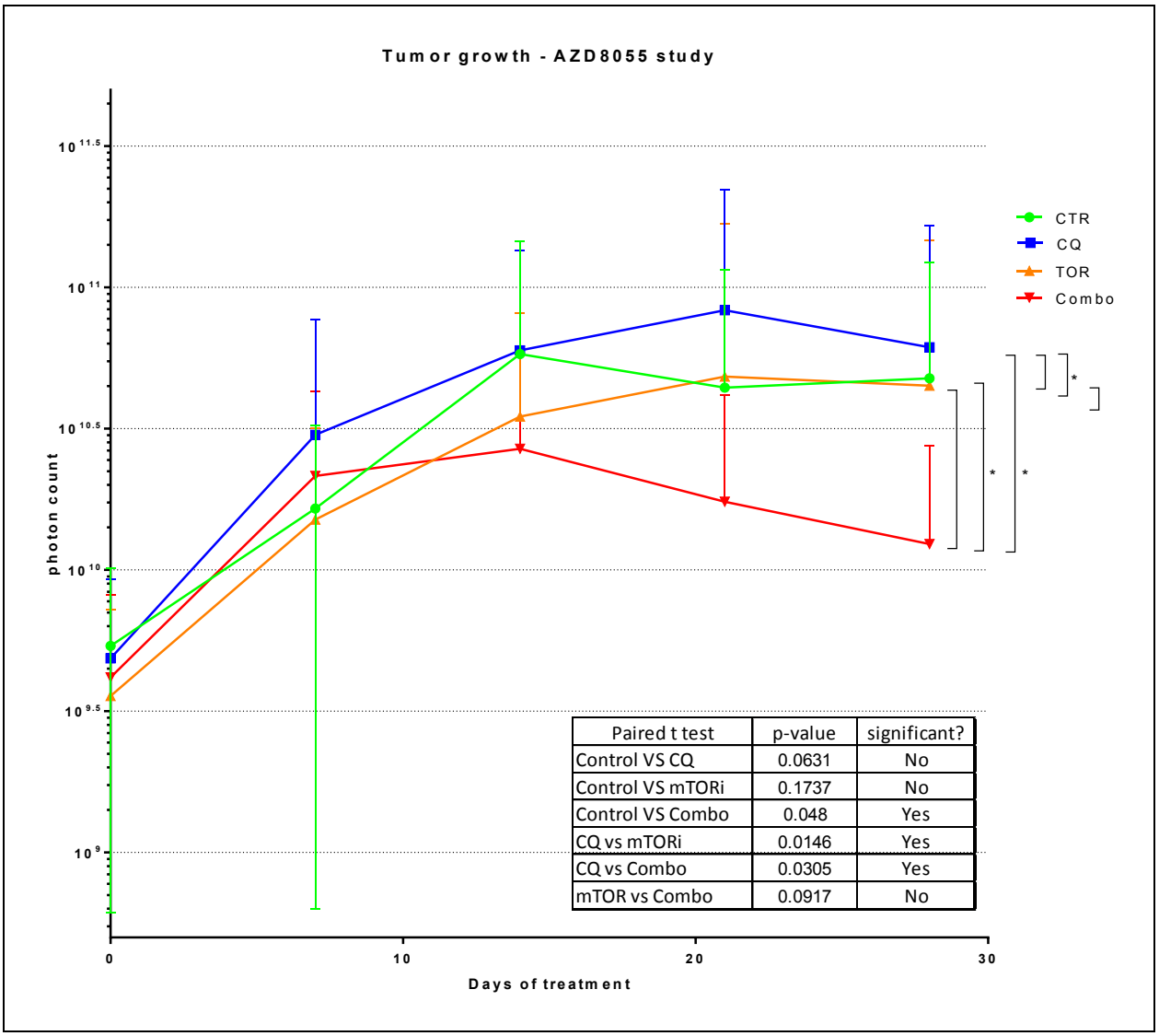


Figure 4. 16: Graph showing the tumor growth estimated by photon count of a 4 arms study over 28 days. Student's t-test showed significant difference between Control VS Combo, AZD8055 VS Chloroquine, and Chloroquine VS Combo but not between AZD8055 VS Combo, Control VS Chloroquine and AZD8055 VS Combo.

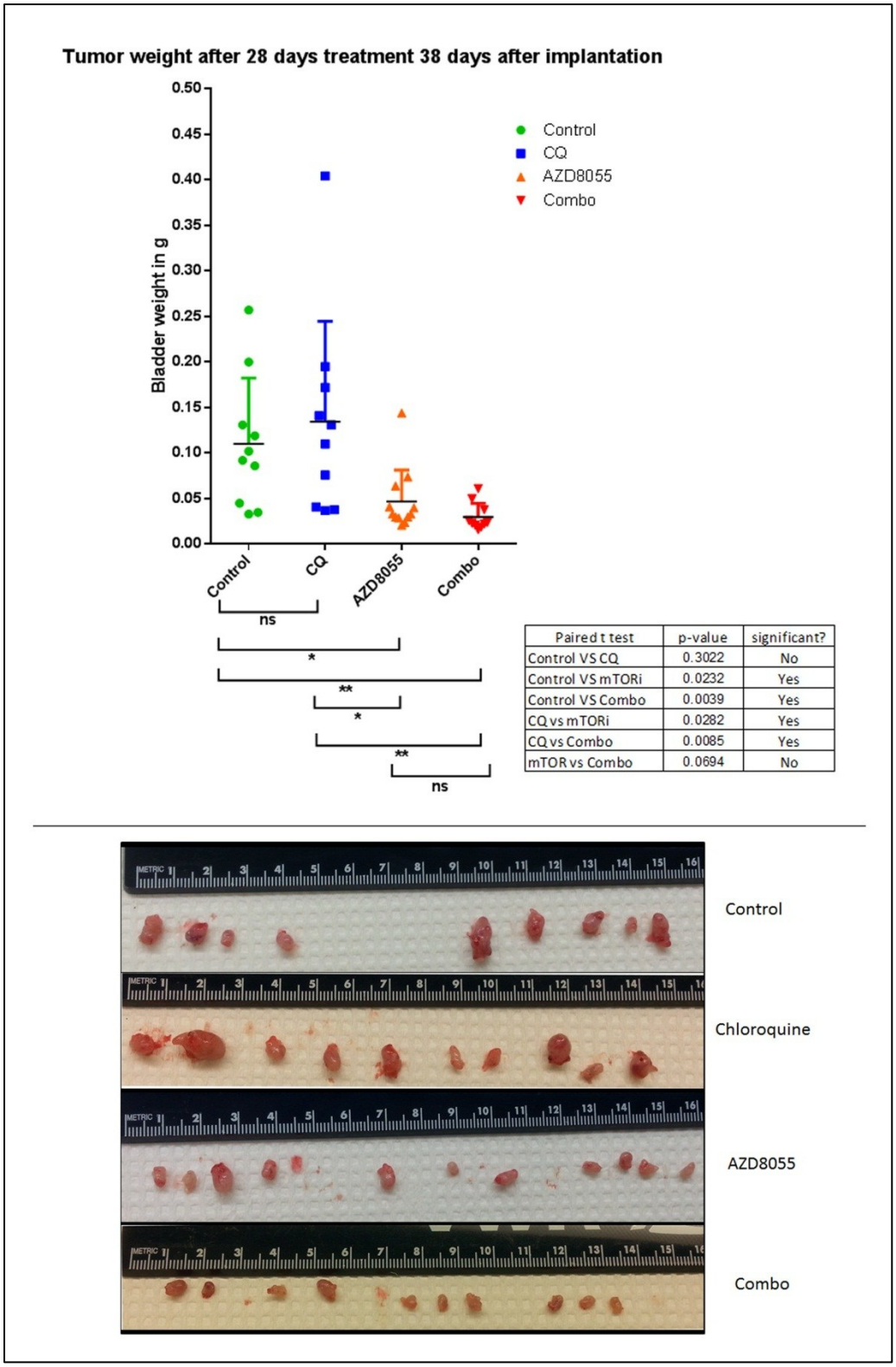


Figure 4.17. Graph showing the comparison of bladder weight in a 4 arms study over 28 days. Student's t-test showed significant difference between Control VS AZD8055, Control VS Combo, Chloroquine VS AZD8055, and Chloroquine VS Combo but not between AZD8055 VS Combo, Control VS Chloroquine.

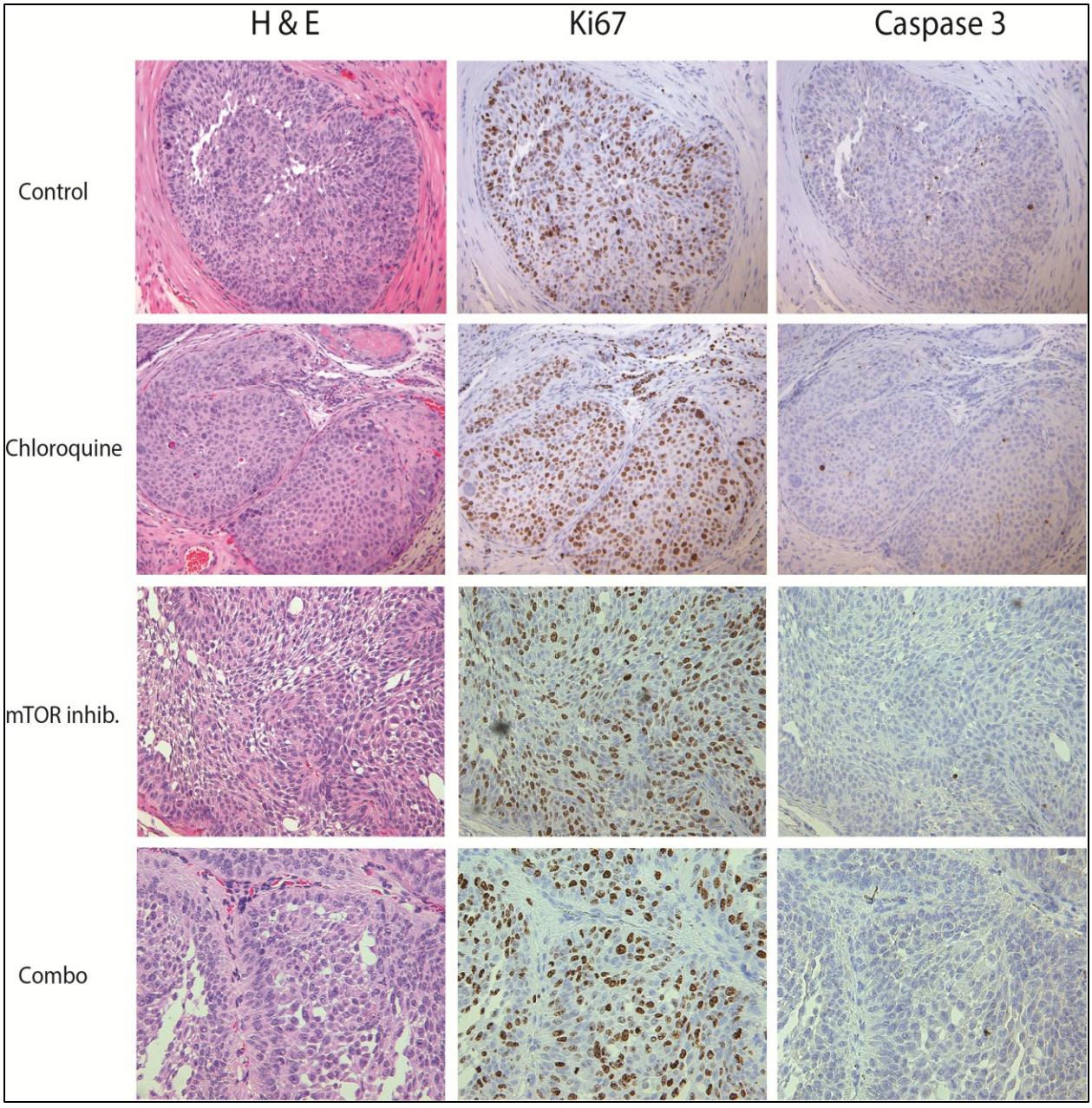


Figure 4.18: The Ki67 and Caspase 3 staining did not show any significant difference between treatment and control groups contrasting with the results shown in figure 4.16 and 4.17.

4.1.6 Block of autophagy coupled with the IGF-1 Receptor inhibitor BMS-754807 induces apoptosis in some bladder cancer cell lines

We already showed how inhibiting the PI3K pathway using a small molecule inhibitor directed against AKT and mTOR would activate autophagy (164). Because IGF-1R regulates,

among others, the same pathway, I decided to test the ability of BMS-754807 to induce autophagy in bladder cancer cells, studying its effects on 2 resistant, 2 intermediate and 3 sensitive cell lines (Fig.4.19A). I ran LC3 western blots and estimated activation of autophagy by the ratio between LC3II/LC3I, estimated by densitometry, as described in other publications (199). Interestingly, I noticed how our IGF-1R inhibitor only activated autophagy in sensitive cells but not in intermediate or resistant cells, suggesting a correlation between drug sensitivity and autophagy. I confirmed these data by LC3 immunofluoresce (197) using an autophagy activating cell line (UC14) and a cell line that did not activate autophagy (T24) (Fig.4.19B). Similar to what was observed in Figure 4.19A, UC14 showed a dose dependent increase in the LC3 punctae compared with T24, in which the LC3 fluorescence stayed low. These data suggest that there may be a correlation between growth factor receptor sensitivity and activation of autophagy as a cytoprotective mechanism. Notably, the consistent resistance of T24 to autophagy activation upon exposure to all 3 inhibitors utilized in this thesis shows how this type of reaction to PI3K/AKT pathway inhibition is not common to all types of bladder cancer and should be kept in mind in light of possible future clinical trials. Moreover, the mechanism behind T24's lack of autophagy activation could have several possible explanations: the simplest of those is the presence of possible inactivating mutations/deletions in regulatory genes for autophagy or alternatively activating mutations/amplifications in suppressor gene for autophagy. A biological reason for those mutations could be a potential selective advantage in shutting off autophagy that, as previously mentioned could be an alternative cell death mechanism, therefore armful for tumor development. An alternative explanation could be that because autophagy is activated by cell starvation, rebound effects deriving from PI3K pathway inhibition may activate pro-metabolic pathways, like for example the MAPK pathway. In this case, the pro-autophagy effects

derived by our drugs would be counterbalanced by the activation of those pathways. A third possibility is that in cells like T24, autophagy is mainly regulated by other pathways different from the mTOR pathway. Some examples are the eIF2α/AMPK pathway responding mainly to starvation, ER stress and d/s RNA or like the IP_3 receptor pathway. Further experiments would be needed to address this hypothesis.

The fact that BMS-754807 exposure in certain bladder cancer cells resulted in autophagy activation suggests autophagy to be a possible cytoprotective response to the inhibitor, as also shown by recent studies (195,196). Therefore, I investigated the effects of chemical autophagy inhibition in combination with the IGF-1R inhibitor, hypothesizing that autophagy activation could be the reason for the lack of drug induced cell death. I ran a cell cycle analysis, exposing 3 autophagy activating cells (UC14, UC13 and RT112) and 3 resistant cell lines that did not activate autophagy (UC16, J82 and T24) to BMS-754807 in single or in combination with the chemical inhibitor of autophagy, Chloroquine disolphate (Fig.4.20). Our data show how all the cell lines displaying activation of autophagy in Figure 3.18 had a relevant increase in apoptosis when exposed to IGF-1R inhibitor in combination with Chloroquine, even at 0.5 μM. Notably, the major pro-apoptotic effects of the combination were observed between 0.5 and 5 μM, which are considered to be clinically relevant concentrations. Relevant effects were not observed, on the other hand, in resistant cell lines.

Finally, I sought to confirm that the pro-apoptotic effects observed with Chloroquine were indeed due to autophagy inhibition and not due to an off target effect of the drug; so I silenced the expression of ATG5 and ATG7, two of the main proteins regulating autophagy, (106) and tested a sensitive and resistant cell line (UC14 and UC16) at increasing concentrations of BMS-754807 (Fig.4.20). Our data confirmed how, similar to what was observed in Figure

4.21, the inhibition of autophagy by ATG5 and ATG7 KD in UC14 led to an increase in autophagy when exposed to 1 and 10 μM of the IGF-1R inhibitor, while lower or no effects were observed by the inhibitor on WT or NT UC14. Once again, the major beneficial effects were observed at the lower concentrations. In UC16, I also confirmed that inhibition of autophagy did not improve the cytotoxic potential of the IGF-1R inhibitor.

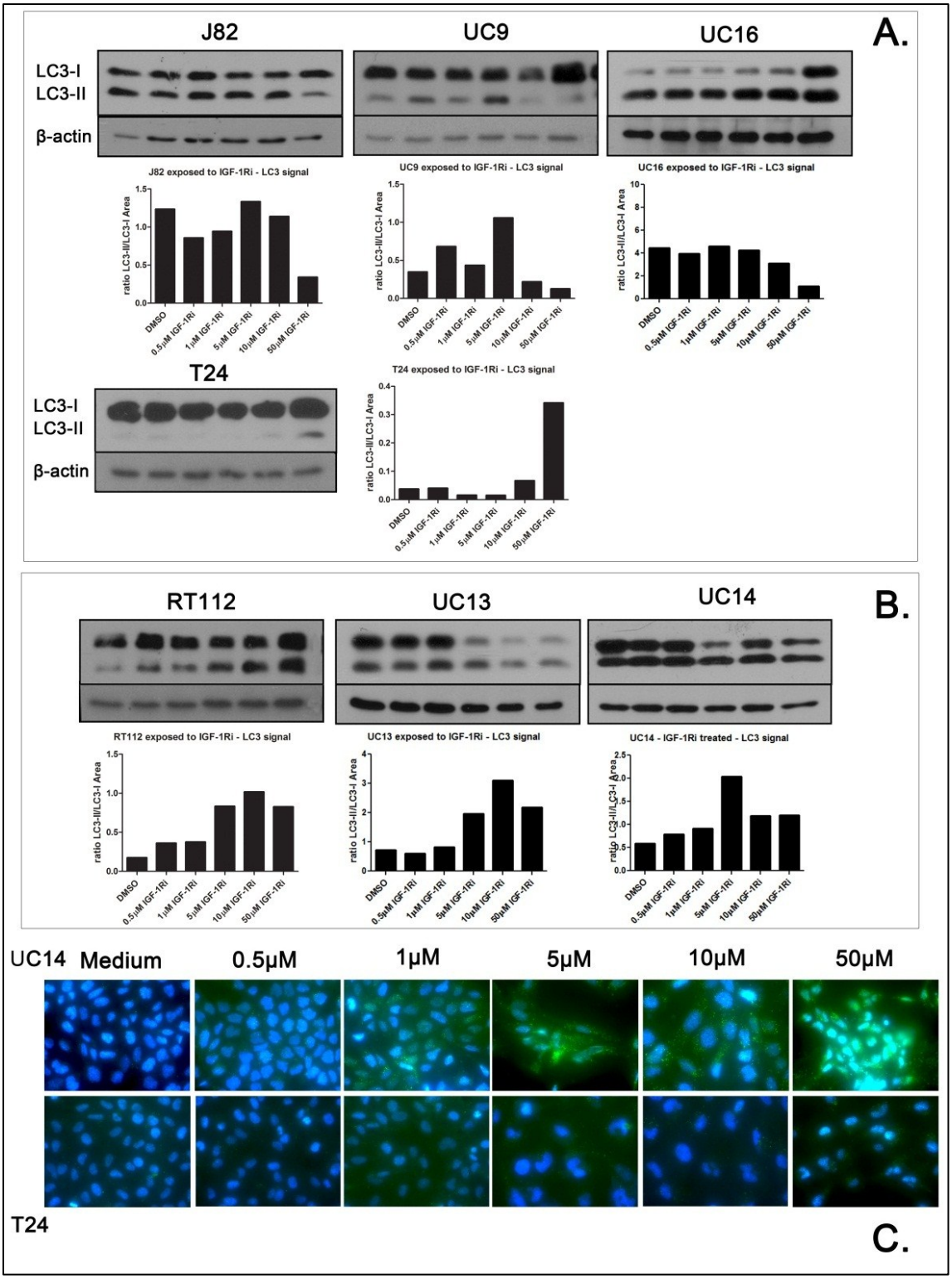


Figure 4.19: Concentration-dependent effects of BMS-754807 on autophagy. Immunoblot displaying LC3-I and LC3-II expression in four autophagy non-activating cell lines: J82 UC9, UC16 and T24 (A) and 3 autophagy activating cell lines: RT112, UC14 and UC13 (B). The LC3 bands were quantified using Image J software and the bar graphs show the ratio of LC3-II to LC3-I as a function of autophagy. (C) shows immunofluorescence analysis of LC-3 localization in UC14 and T24 cells.

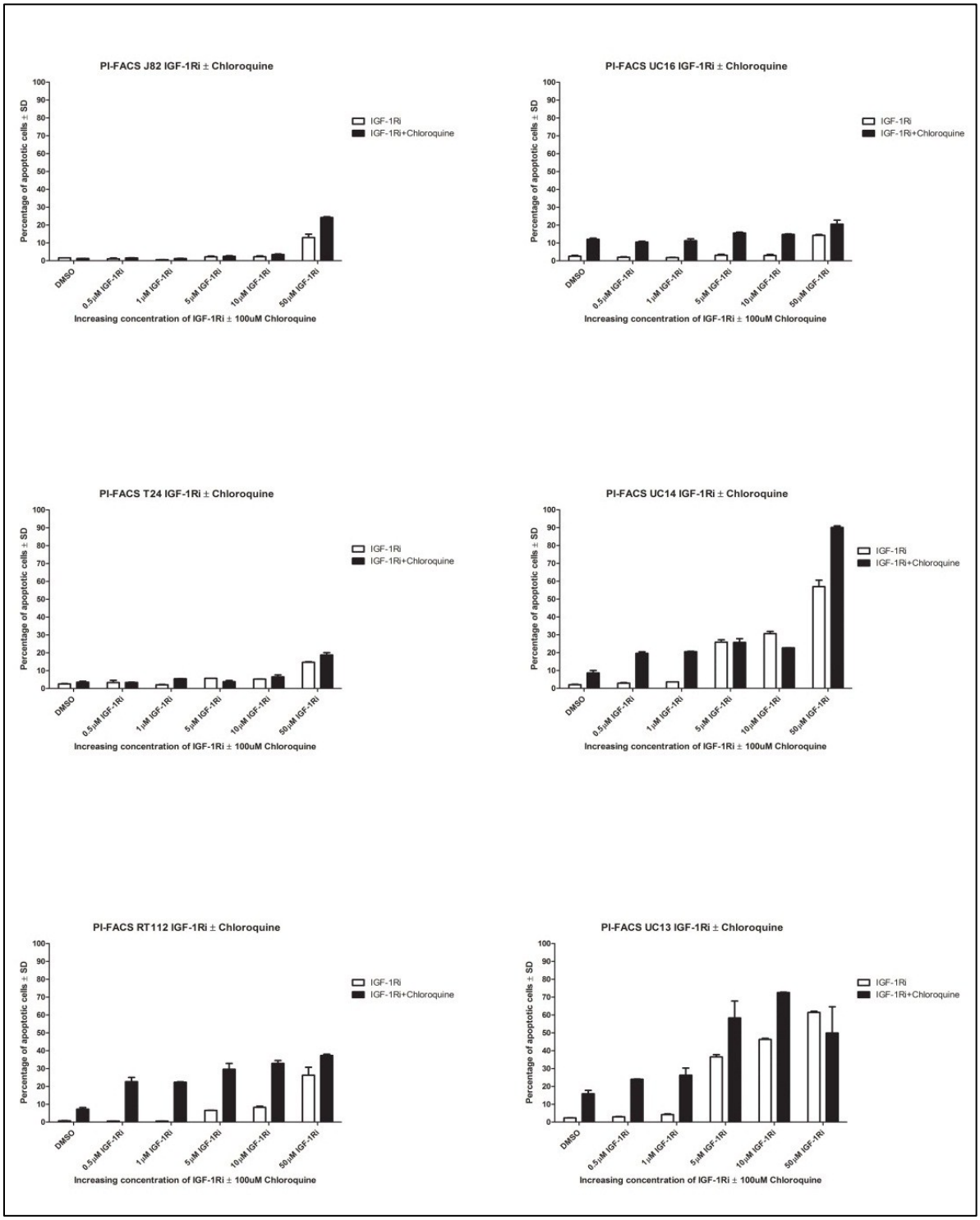


Figure 4.20: Effect of BMS-754807 on apoptosis, as single agent or in combination with chloroquine. Bladder cancer cells (J82, UM-UC-16, UM-UC-14, RT112, UM-UC-13 and T24) were exposed to increasing concentrations of BMS-754807 alone or in combination with a fixed dose of 100 μM chloroquine. Apoptotic cells were quantified by PI-FACS.

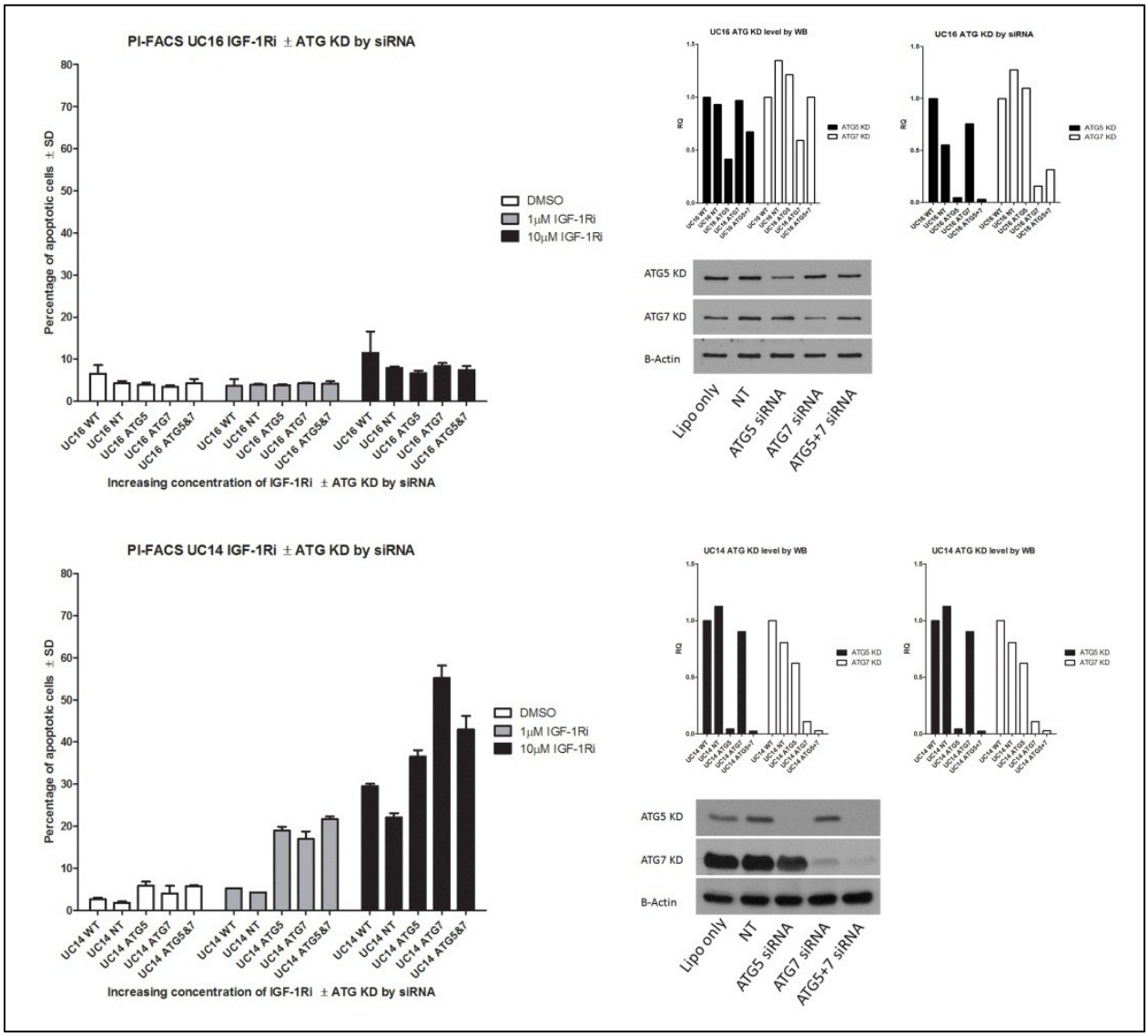


Figure 4.21: Effects of BMS-754807 on ATG5 and ATG7 knock down cells UC14 and UC16. UC14 is an activator of autophagy when normally exposed to BMS-754807, while UC16 did not show any autophagy activation

CHAPTER 5: DIFFERENTIAL MODULATION OF DOWNSTREAM PATHWAYS TO GROWTH FACTOR RECEPTORS IN IGF-1R DEPENDENT CELL LINES

While investigating the antiproliferative effects of BMS-754807 on bladder cancer cells I noticed how almost all the sensitive cells were also dependent on either EGFR or FGFR (Figure 5.1). Because IGF-1R, EGFR, and FGFRs are all thought to regulate the same downstream pathways, I was curious to find out why cells would be dependent on two of them. Previous studies have shown how FGFR and EGFR, for example, counterbalance each other. The inhibition of one of them increases the expression of the other, but cells are generally never dependent on both at the same time (200). When I tested the on-target effects of BMS-754807, I noticed that the inhibitor had a relevant dose-dependent effect on AKT phosphorylation but little to no effect on ERK phosphorylation. These observations led me to hypothesize that cells that displayed dependency on more than one growth factor receptor had indeed different regulation of the two main downstream pathways PI3K and MAPK. IGF-1R would better regulate the PI3K pathway while FGFR in the case of RT112 would better regulate the MAPK pathway. I tested this hypothesis on 3 different IGF-1R dependent cell lines: RT112 and UC14 (reported in literature to be also dependent on FGFR) and UC6 (reported to be dependent on EGFR) (188,201-204). In all of these cell lines BMS-754807 showed a time-dependent inhibition of AKT phosphorylation, but little or no effects on ERK phosphorylation confirming the data shown in figure 3.12. Partial or complete inhibition of ERK phosphorylation was instead shown when UC14 and RT112 were exposed to the FGFR inhibitor AZ4547 but not when exposed to the EGFR inhibitor Iressa. Vice-versa, in UC6 ERK inhibition was detected only when the cells were exposed to EGFR inhibitor, but not when exposed to the FGFR inhibitor. The fact that the IGF-1R inhibitor did not have any effect on ERK phosphorylation in any of the tested cells confirmed our initial hypothesis that IGF-1R mainly regulated the PI3K pathway in sensitive cells. No noteworthy effects were observed on S6 phosphorylation at 1, 3, and 6h perhaps

because its dephosphorylation takes longer than 6h to be detectable. Figure 3.12 in fact, showed a dose-dependent inhibition of S6 phosphorylation at 24h where RT112 was exposed to BMS-754807.

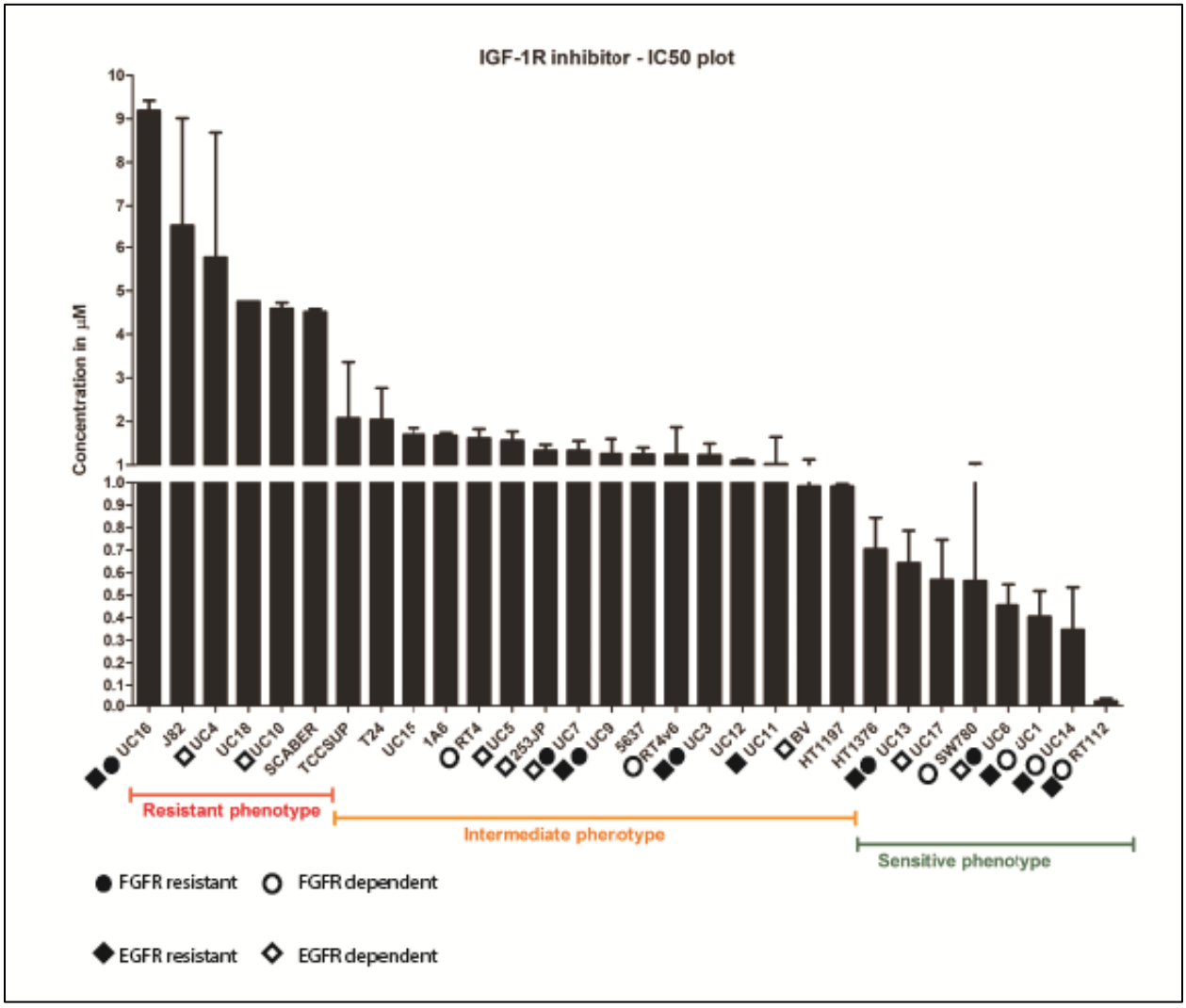
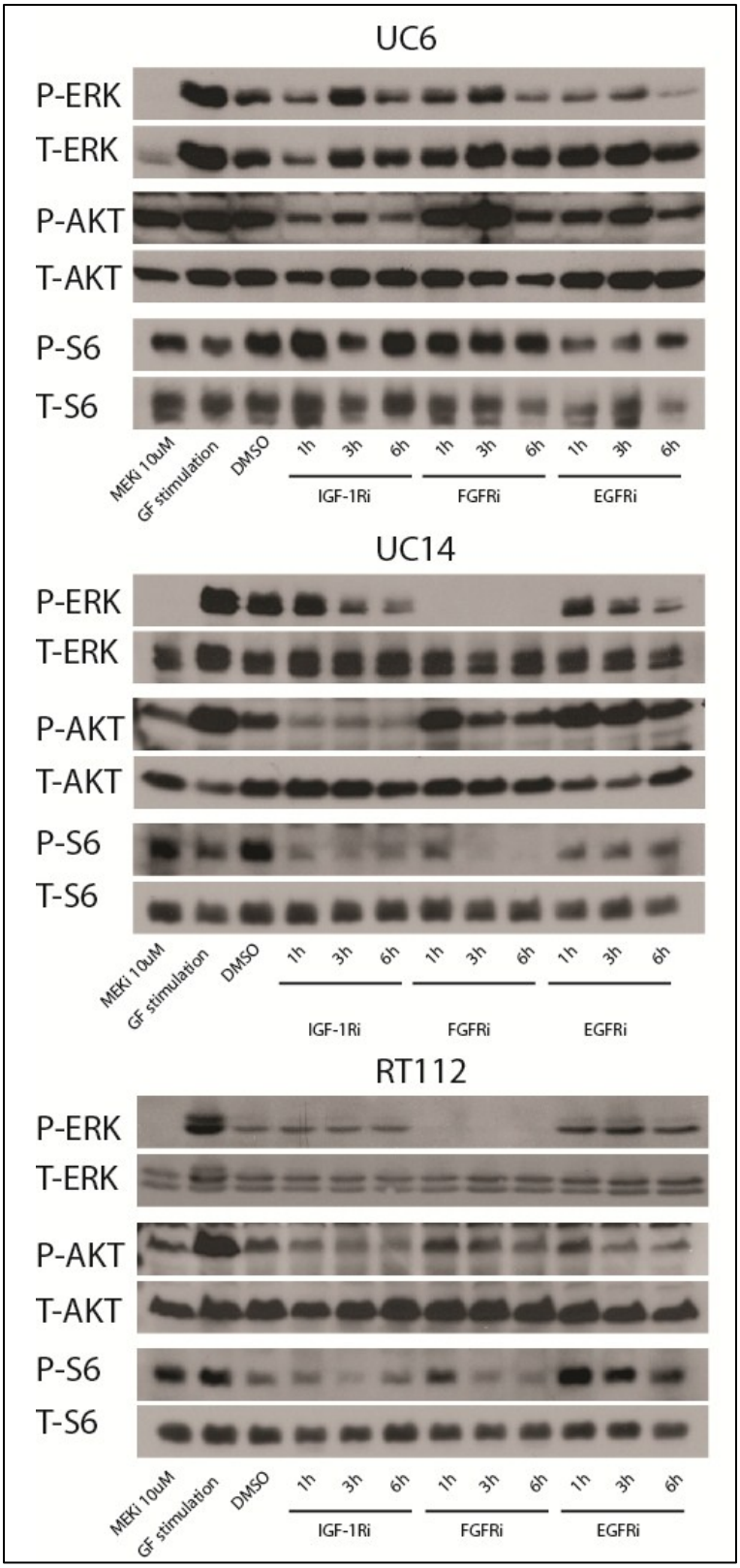


Figure 5.1: The IC50 waterfall plot shows the dependency of bladder cancer cells on other growth factor receptors other than IGF-1R. Noteworthy is the fact that almost all of the sensitive cell lines are also dependent on either FGFR or EGFR.


UC6
P-ERK
T-ERK
P-AKT
T-AKT
P-S6
T-S6
MEKi 10uM
GF stimulation
DMSO
1h
3h
6h
1h
3h
6h
1h
3h
6h
IGF-1Ri
FGFRi
EGFRi
UC14
P-ERK
T-ERK
P-AKT
T-AKT
P-S6
T-S6
MEKi 10uM
GF stimulation
DMSO
1h
3h
6h
1h
3h
6h
1h
3h
6h
IGF-1Ri
FGFRi
EGFRi
RT112
P-ERK
T-ERK
P-AKT
T-AKT
P-S6
T-S6
MEKi 10uM
GF stimulation
DMSO
1h
3h
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1h
3h
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3h
6h
IGF-1Ri
FGFRi
EGFRi

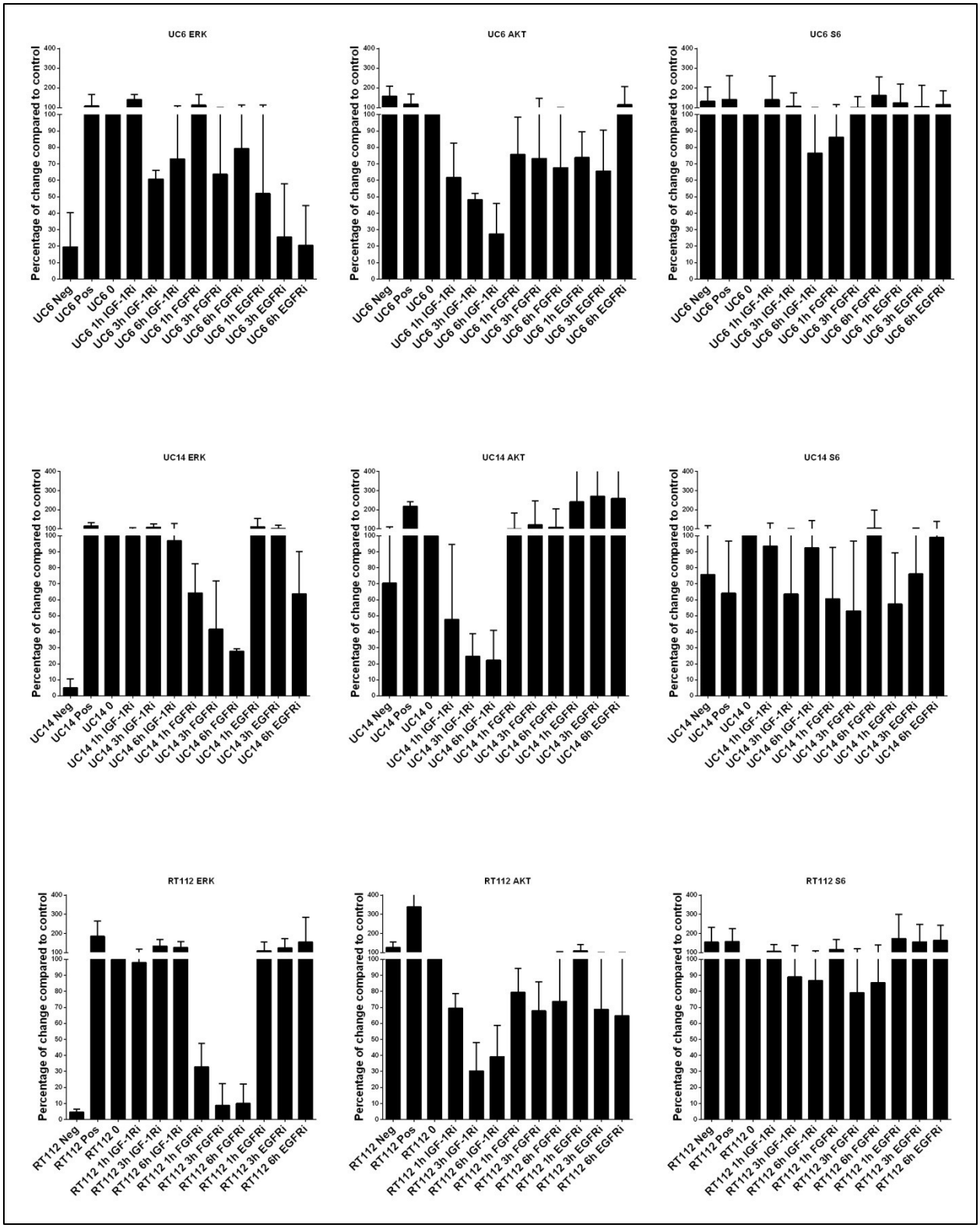

Figure 5.2: Western blots show the effects of IGF-1R, FGFR and EGFR inhibitors on the downstream targets of UC6, UC14 and RT112. Bands are quantified and plotted into bar graphs.

The fact that sensitive cells to the IGF-1R inhibitor were also sensitive to either EGFR or FGFR inhibitors made us wonder whether combining the two growth factor receptor inhibitors would have any significant anti-proliferative effect. I then tested EGFR dependent cell lines UC16, UC6 and FGFR dependent cell RT112 and SW780 in MTT assay (Figure 5.5 and 5.4). I combined the respective IC50 concentration of IGF-1R inhibitor with increasing doses of FGFR or EGFR inhibitor. I then observed at least additive effects when BMS-754807 was combined with FGFR on RT112 and SW780 cell or EGFR on UC16 and UC6. Surprisingly, the addition of Iressa on SW780 cells and AZD4547 on UC6 and UC16 completely counteracted the anti-proliferative effects of BMS-754807 and resulted in increased cell growth.

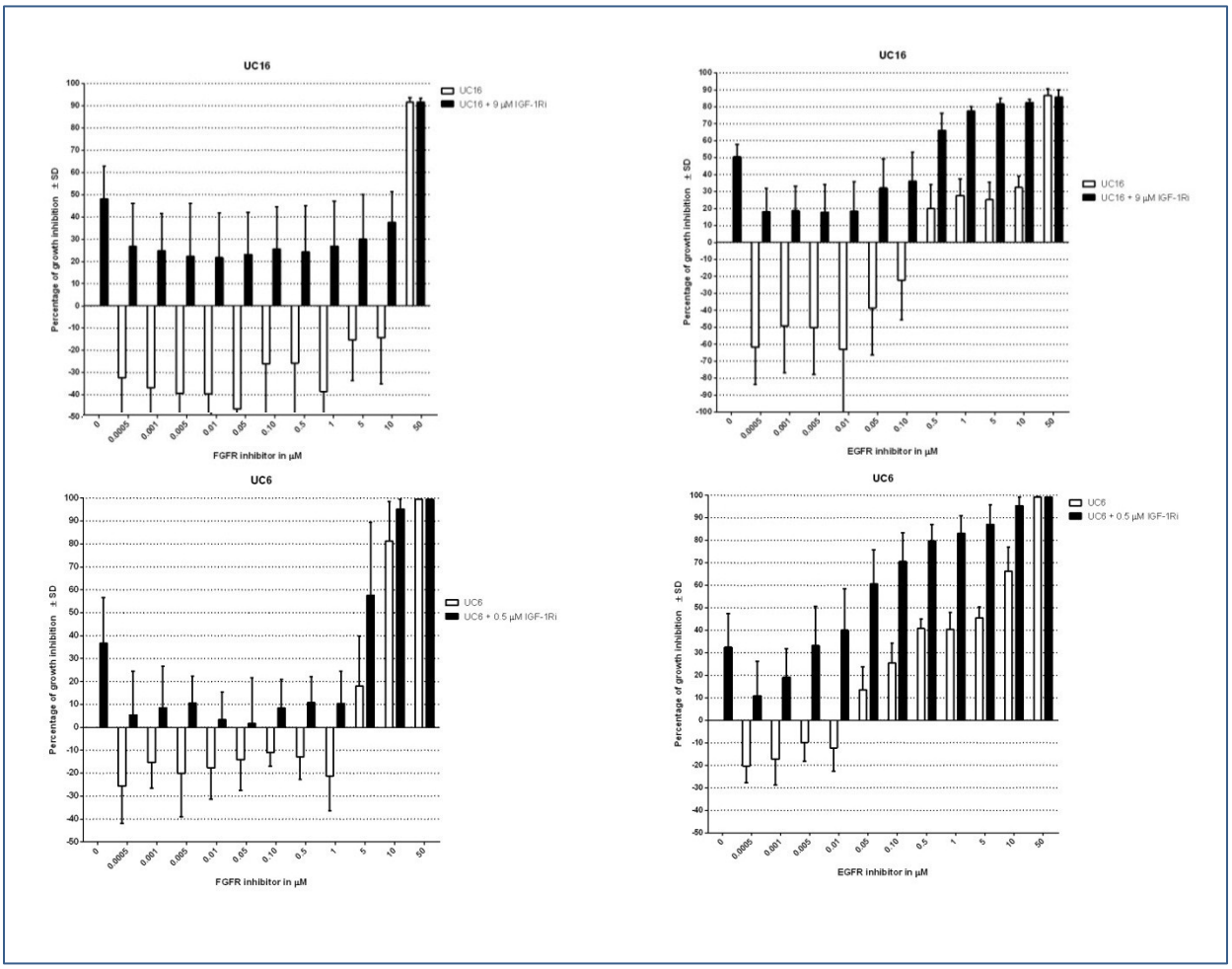

Figure 5.3: Effects of FGFR and EGFR inhibitor alone or in combination with IGF-1R inhibitor on EGFR dependent cells UC6 and UC16

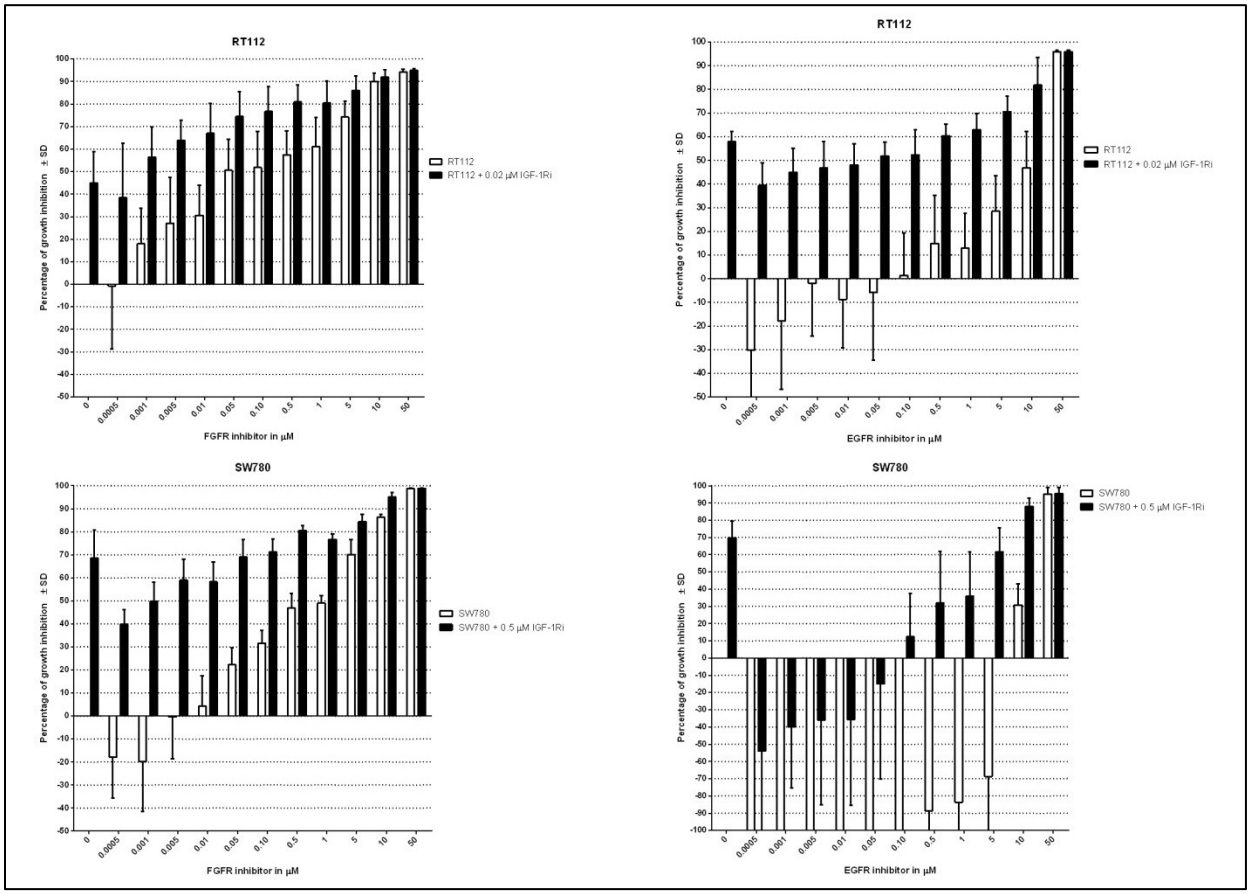

Figure 5.4: Effects of FGFR and EGFR inhibitor alone or in combination with IGF-1R inhibitor on FGFR dependent cells RT112 and SW780

Chapter 6. DISCUSSION & FUTURE DIRECTIONS

6.1 The missing link between genetic signature and predictability.

6.1.1 Link between genetic signature and sensitivity to AZ7328

After many years of talking about personalized medicine as the best way to approach cancer therapy, the discovery that non-small cell lung cancer carrying mutated EGFR was particularly responsive to Gefitinib treatment, generated great enthusiasm in the scientific community (205-208). A few years later it was reported how metastatic melanomas carrying the E600V activating BRAF mutation was particularly sensitive to Plexxicon (209-213), confirming the idea that the genetic signature of a tumor should dictate the best targeted therapy to be used in each specific case, allowing us to easily predict the outcome of a therapy before its start.

Having this idea in mind, I began my study in the effort of finding a link between the mutation status of our bladder cancer cell lines and the sensitivity to the AKT inhibitor AZ7328. As mentioned in the introduction of this thesis, many well-known mutations are present in bladder cancer that may promote the activation of AKT: FGFR3, PIK3CA, RAS and PTEN. I therefore focused on the investigation of these proteins to assess an eventual correlation between AKT sensitivity and mutation status. Based on what was shown in Table 1.1, J82, UM-UC-6, UM-UC-14 and UM-UC-16 have mutations in FGFR3. T24 and UM-UC-6 showed c-MET mutations, while 253J B-V, J82, UM-UC-3, UM-UC-5, UM-UC-6 and UM-UC-16 had PIK3CA mutations. Fincally, T24 and UM-UC-3 showed RAS mutations, UM-UC-5 had EGFR amplification, while J82 and UM-UC-3 displayed PTEN deletions. Contrary to our initial expectation only a loose correlation between sensitivity to AKT inhibition and the presence of activating PIK3CA mutations was found. In paragraph 1.2.2 I showed how PIK3CA has been

reported to have 3 main recurring mutations: H1047R, E545K and E542K. The first 2 were identified in 10 out of 12 of our bladder cancer cell lines. Mutations in the residue E545K cause the disruption in the inhibitor interaction between the two subunits of PI3CA: the regulatory subunit p85 and the catalytic subunit p110 (214). Mutations in residue H1047R induce increased lipid kinase activity, promoting enhanced activity for PIK3CA substrate PIP_2 (215). The third mutation identified only in J82 cells is P124L and is localized in a region of four helices between the adaptor-binding and RAS-binding domains. Its function is still unknown; however, similar mutations have been reported in colorectal cancer, suggesting an activating role for this mutation (216). These observations are important to a potential AKT inhibitor based therapy for bladder cancer as, according with the TCGA data shown in figure 1.3; approximately a third of all bladder cancer patients have PI3CA alterations. The mutations reported here would account for at least 65% of all PIK3CA mutations (77). Because of the tight association between PI3CA mutation and low grade tumor/early stage bladder cancer (11), a therapy based on AKT inhibitors may be useful in preventing recurrence in patients.

Unfortunately, no correlation between AKT inhibitor sensitivity and PTEN expression or activating AKT mutations was observed, suggesting that defining a reliable pattern for sensitivity could be more difficult than previously anticipated. Interestingly, 6 of 7 cell lines displaying high levels of AKT phosphorylation express molecular features of epithelial-to-mesenchymal transition (EMT) (217,218). It is noteworthy to mention how none of our cells were particularly sensitive to AZ7328. UC5, our most sensitive cell line, showed an IC50 of ~1 μM, while for all the other tested cells much higher doses were necessary to reach a satisfactory growth inhibition. Even at the high concentration of 5 μM, only UC5 and UC6 reached their IC50. It is reasonable to assume that any growth inhibitory effect beyond that concentration is just the result of an off

target effect of AZ7328. Even the western blots in paragraph 3.3 show how, with the exception of UC5 and UC6, the phosphorylation level of downstream targets of AKT like P-GSK needed concentrations of the drug higher than 1μM to show any appreciable effect. It is reasonable to hypothesize that the effect of AZ7328 on bladder cancer cells might have been not specific enough to truly fulfill the needs of a study that aimed to link genetic signatures with patterns of resistance. Perhaps a full genome sequencing and a higher number of screened cell lines could have been more helpful in finding key predicting mutations. Another important consideration is the similar pattern of sensitivity to the one observed with AZ7328 was also observed with the double mTOR inhibitor, with the exception of the outlier UC3 and UC13 cells. Surprisingly rapamycin had a completely different pattern of sensitivity, suggesting that the one observed with AZ7328 and AZD-4264 was mainly determined by the inhibition of AKT phosphorylation.

6.1.2 Correlation between IGF-1R expression and sensitivity to BMS-754807

Our next step in characterizing pattern of sensitivity to small molecule inhibitors was to investigate a possible correlation between IGF-1R expression and sensitivity to IGF-1R inhibitor. The insulin-like growth factor (IGF) signaling system is involved in the regulation of growth and metabolism and has a critical role in the development of various tissues (219,220). IGF-1R, a tyrosine kinase receptor, is expressed in many human tissues and regulates apoptosis, cell differentiation, migration, cell growth, and induces autophagy. Recent research indicates that deregulation of the IGF signaling pathway, and overexpression of IGF-1 receptor in particular, occurs in several types of cancer, including prostate, colorectal, bladder, and breast cancer (219,221), and plays an essential role in cancer cell proliferation and metastasis (222-226). IGF-1R has also been shown to promote motility and invasion of bladder cancer cells. Recent studies suggest that there may also be a correlation between deregulation of this pathway and an

increased risk of developing cancer (22,220,227). I started this study by testing the efficacy of the IGF-1R inhibitor BMS-754807 in a panel of 30 bladder cancer cell lines, as previous studies have shown the importance of this growth factor receptor in urothelial cancer (221,228-231). I started evaluating the anti-proliferative effects of blocking IGF-1R, being able to discriminate between 3 different phenotypes: sensitive (IC50 < 1 μM), intermediate (1 μM< IC50< 2 μM) and resistant (IC50 > 1 μM). In our experience, the majority of these small molecule inhibitors have mostly cytostatic but not cytotoxic effects on bladder cancer (164,188,189). Therefore, I tested the cells by cell cycle analysis confirming that the anti-proliferative effect of BMS-754807 was mostly due to cell cycle arrest. Next I tried to correlate IGF-1Ri sensitivity to receptor expression failing to find any correlation between the two. The only cell line that perhaps owes its resistance to low IGFR expression is J82 whose IGFR levels are undetectable. RT112, our most sensitive cell line and UC 16 our most resistant cell line showed average levels of IGF-1R expression. The western blot testing the on-target effects of BMS-754807 showed how little or no effect on AKT and S6 phosphorylation was observed in UC16 cells, while a more significant effect was detected for ERK phosphorylation. However, these results were observed exclusively under regular FBS media and IGF-1 induced media, suggesting that the presence of IGF-1 in the media is necessary to detect any BMS-754807 activity. Moreover, the fact that, despite a ~50% drop in ERK phosphorylation, UC16 showed very limited growth inhibition at 0.1 and 1 μM, suggests that in this cell line the PI3K pathway is more important than the MAPK pathway in driving proliferation. Interestingly, in RT112, our most sensitive cell line, the inhibitory effects of BMS-754807 were only observed in P-AKT and P-S6 but not in P-ERK under all 3 media conditions (FBS, serums starved, and IGF-1 induced conditions), suggesting that in this cell line IGF-1R mainly regulates the PI3K pathway. These observations led us to hypothesize that IGF-1R in

sensitive cell lines specifically affects the PI3K pathway while the regulation of the MAPK pathway is perhaps driven by some other growth factor receptor. This hypothesis is discussed in more detail in chapter 5 of this thesis and later in this discussion section.

Finally, no correlation was observed when comparing the IGF-1R pattern of sensitivity to molecular alterations in bladder cancer cell lines. PIK3CA activating mutations are found in resistant cells like UC16 and J82 as well as in sensitive cells like UC6 or even intermediate sensitivity cells like UC3 and UC5. Similarly mutations/ amplifications in other growth factor receptors like c-MET, EGFR and FGFR are also found in cell lines scattered throughout the whole IGF-1R inhibitor's waterfall plot. This could be explained by the fact that IGF-1R and FGFR/EGFR better regulate different signaling pathways, as described in chapter5. PTEN alterations as well as AKT differential baseline phosphorylation also did not show any correlation with sensitivity to BMS-754807.

The disappointing results obtained by the AKT and IGF-1R study in trying to correlate response to small molecule inhibitors with the genetic signature of the tumor suggest that this path may not be as straightforward as the encouraging results with Gefitinib and Plexxicon led us to believe, and that a much deeper knowledge of the genetic signature, epigenetics and cancer cell signaling has to be gained before a real personalized therapy era could begin.

6.2 Autophagy, the gatekeeper to apoptosis.

6.2.1 Strategies to sensitize bladder cancer cells to the AKT inhibitor AZ7328

In this thesis I showed how despite the fact that small molecule inhibitors targeting the PI3K pathway induce cell death in many types of cancer (175,232,233), in bladder cancer they only seem to have cytostatic but no cytotoxic effects. In the effort to enhance AZ7328 pro-

apoptotic activity I combined it with conventional chemotherapeutic agents, like TRAIL and gemcitabine/cisplatin. Unfortunately, no effects whatsoever were observed in any of the tested cell lines (UC9, UC5 and 253J B-V). I then combined AZ7328 with the TORC1 inhibitor rapamycin, which resulted at least in additive effects on growth inhibition in 11 of 12 cell lines, demonstrating that double inhibition of the same pathway could be beneficial in slowing down tumor growth.

One of the main effects of the PI3K pathway in the presence of activating nutrients is the suppression of autophagy (196). Autophagy as mentioned in chapter 1.3 is a potential pro-survival catabolic process activated upon cell stress and starvation and many studies have associated this process with enhanced resistance to cytotoxic effects in several types of cancer treatments (195,196). Disappointed by the lack of cytotoxic activity that AZ7328 showed on our cells, I speculated that autophagy activation might be responsible for inhibiting drug-induced cell death. Indeed, our data showed how AZ7328 induced concentration-dependent autophagy activation in three out of the four tested cell lines. The block of this crucial cellular process by chemical inhibitor Chloroquine finally induced dose-dependent cell death. These positive results encouraged us in testing autophagy inhibition in combination with other small molecule inhibitors, investigating its effects in both *in vitro* and *in vivo*. In paragraph 4.1.5, I discussed how T24 was consistently resistant to autophagy activation, proposing 3 different hypotheses to explain this observation: 1) alterations in autophagy regulatory genes, 2) activation of alternative pro-metabolic relief pathways (MAPK pathway) and 3) alternative regulation of autophagy by other pathways (IP_3R, AMPK etc.). To further investigate this issue, I propose a series of future experiments. To address hypothesis 1), it would be sufficient to sequence T24 to identify the presence of possible mutations in key regulatory genes for autophagy. A good expression

profiling analysis by microarray along with gene sequencing would allow me to address eventual amplifications or deletions. To address hypothesis 2), I could exposed T24 cells to one of the small molecule inhibitors targeting the PI3K pathway (e.g. AZD8055) for 24/48h and compare a microarray/RPPA analysis to unexposed T24 cells. This comparison would highlight those genes that are upregulated upon AZD8055 exposure, allowing me to verify whether some relief pathway is indeed activated or not. The validation of hypothesis 3 would require a little bit more work, I would have to selectively knock down key genes of the alternative regulatory pathways for autophagy activation and subsequentially serum-starve the cells. LC3 punctae immunofluorescence would allow me to identify the most important pathways for activation of autophagy in T24 cells.

6.2.2 Strategies to sensitize bladder cancer cells to mTOR inhibitors

AZD-4264 and its clinical candidate AZD-8055, are two novel mTOR inhibitors able to target both the mTOR complexes TORC1 and TORC2 (167,168), in comparison to older drugs like rapamycin and RAD001 that only targets TORC1. In the beginning I was very excited to test the effects of these drugs on bladder cancer cells. Unfortunately, similar to what was observed with AZ7328 and despite good specificity and anti-proliferative effect, I did not detect any cytotoxic effect in the tested cell lines, greatly lowering its translational benefits for patients. Even combinations of AZD-4264 with conventional chemotherapy did not show any improvement in the levels of cell death. On the other hand, I found that both drugs were able to induce potent autophagy activation in some of the tested cells, confirming previous observation in different types of cancer. (168,169). I tried to confirm our hypothesis that autophagy is one potential mechanism that bladder cancer cells use to escape apoptosis, and that blocking this process would make AZD-4264 and AZD-8055 cytotoxic to some cell lines.

In this study I was able to inhibit autophagy activation both chemically and genetically by using Chloroquine or siRNA targeting ATG5 and ATG7, two key regulatory genes for this process (see introduction). The addition of 1 and 10 μM of AZD-4264 or AZD-8055 was able to induce a significant increase in cell death in 2 out of 4 cells lines tested. Notably, both the cell lines that showed an increase in cell death, activated autophagy upon exposure to AZD-4264 or AZD-8055. As discussed in chapter 4, despite a significant increase in the induction of apoptosis, only 50% of cell death was achieved at clinically relevant concentrations. The study of the escaping mechanisms behind it, could represent a fascinating project in itself. A tempting future strategy to address this issue could be the orthotropic implantation of bladder cancer cells in xenograft models in a two arms study. A group would be used as a control and another would be treated with a combination of AZD8055 and Chloroquine. The treatment of the latter group would go on until resistance would arise. At that point, I would harvest the tumors and generate a heat map genetic profile of both groups and compare them. Once identified potential mechanisms of resistance, I would knock down key genes of such mechanisms by using inducible constructs and repeat the same exact animal study proposed above. This time I would keep the knock down construct off until resistance would arise and then turn it on. If my hypothesis is correct, the tumors would start regressing again, confirming the targeted cellular process as the one responsible for the resistance to AZD8055/Chloroquine therapy.

In the next step of my study, I sought to test our approach in xenograft models in order to confirm the good results obtained with the combination of Chloroquine and AZD8055 as well as to assess possible toxicity for the mice. At the end of the study I was able to show an overall significant difference between Control and Chloroquine groups and the mTORi and Combo group. Unfortunately, no difference was detected between AZD8055 alone and in combination

with Chloroquine even though the p-value was very close to be significant (0.069). One of the possible reasons for this result is that the great effect of the mTOR inhibitor alone was effective enough to minimize an appreciable beneficial effect of the combination or that as previously mentioned, estimation by bladder weight is not precise enough when it comes to discriminate between small size tumors. . The histological exams on the tumors did not show any difference in Ki67 staining, and will be repeated using a different proliferation marker. Similarly, results obtained by Caspase 3 staining did not show any apoptosis. I am planning to confirm that performing a TUNEL staining as well. Finally, it is noteworthy to mention that in our hands, AZD8055, alone and in combination with Chloroquine, showed some toxic effects in mice including weight loss, hunchback posture and dermatitis. A diet richer in nutrients and sugar seemed to mitigate such effects allowing us to complete the study.

6.2.3 Strategies to sensitize bladder cancer cells to IGF-1R inhibitors

The fact that BMS-754807 did not induce a significant increase in DNA fragmentation despite the fact that the IGF-1 receptor regulates many important survival processes, suggested that bladder cancer cells had some kind of protective mechanism that would allow them to escape cell death. Encouraged by the positive results obtained from the AKT and mTOR inhibitor studies, I decided to adopt the same approach with BMS-754807, as the IGF-1 receptor regulates, among others, the very same PI3K pathway. Similar to what was done in our previous study I showed that our IGF-1R inhibitor was able to induce autophagy activation in a dose-dependent fashion in some of our cell lines by LC3 western blot and LC3 punctae immunofluorescence. Interestingly, all the autophagy activating cell lines (RT112, UC13 and UC14) were classified as sensitive cells by MTT assay, while J82, UC9, UC16 and T24 which did not show any autophagy increase, all belonged to the intermediate/resistant phenotype. It is

tempting to speculate that bladder cancer cell lines only activate autophagy as a cytoprotective mechanism when they particularly rely on the PI3K pathway to proliferate or survive, but this hypothesis would need further investigation.

In an effort to sensitize the cells to BMS-754807, I blocked autophagy chemically and genetically by combining the inhibitor with Chloroquine dislophate or after transiently silencing ATG5 and ATG7. Data showed how these two approaches were able to relevantly increase the level of apoptosis only in those cells that activated autophagy in response to the IGF-1R inhibitor, while having no effects on the other cells.

In conclusion, I showed once again how combining small molecule inhibitors targeting the PI3K pathway with inhibitors of autophagy could be a valid alternative to conventional chemotherapy. The fact that pro-apoptotic effects were observed already at sub-micromolar concentration strengthens the rationale for a therapeutic approach based on the observations described in this thesis. Encouraging results from our studies may convince clinicians to adopt our approach testing it into a phase I clinical trial while on the other hand, inducing pharmaceutical companies to produce improved and more specific drugs than Chloroquine to inhibit autophagy activation.

The finding that autophagy inhibition led to apoptosis in some bladder cancer cell lines upon PI3K pathway perturbation is extremely important in light of future clinical trials. Cell lines that did not activate autophagy in response to PI3K/AKT pathway inhibition did not show any increase in the levels of apoptosis when any of the inhibitors used in this thesis was combined with Chloroquine. This highlights the importance of uncovering the resistance mechanism behind this subset of cells as well as a potential marker that would allow us to

preventively identify them before the beginning of future therapies based on PI3K/autophagy inhibition approaches. Cell lines activating autophagy upon PI3K pathway inhibition also seemed to be consistent across the whole set of small molecule inhibitors used in my research. Interestingly, autophagy activation was a good predictor for cell lines that would undergo apoptosis upon simultaneous inhibition of the PI3Kpathway and autophagy. Despite that, only a subpopulation of the analyzed cells was classified as sub G1 and therefore as apoptotic (40-60%). It is reasonable to anticipate that a reproducible trial in cancer patients would show good cytostatic effects and partial reduction in tumor size but, at the same time, would also lead to relapses at the end of the trial. As mentioned in Chapter 4, an exciting future challenge would be to identify the escape mechanisms rescuing the surviving cellular sub-population from apoptosis. Being able to specifically target this cellular process may allow us to completely obliterate tumor cells inducing complete remissions in patients.

When I started this project about five years ago, cell lines represented the status of the art for experimental therapeutics. Recent important publications like the TCGA study as well as the paper that our group published in Cancer Cell, showed different subsets of bladder cancer that are unfortunately difficult to match with the available bladder cancer cell lines. Moreover, we discovered how, when bladder cancer cells lines are recycled in xenograft models, many drastic changes at molecular level occur. These findings suggest how cell lines are no longer the best model to investigate novel therapies for cancer treatment. In light of the many scientific progresses achieved during these past 5 years, if I had to start my project all over again, I would use completely different strategies. Using high-throughput techniques like inducible RNAi libraries, I would try to identify key genes for proliferation and survival of bladder cancer using patient-derived tumors instead of cell lines, discriminating them in base of their molecular

feature and their co-localization in the TCGA subsets. I would then use xenograft or alternatively organoids to test my candidate genes by targeting them through inducible knock down and small molecule inhibitors. By using this approach, it is reasonable to expect positive results in at least reducing tumor growth, but based on the results from many previous trials, I would also expect the raise of escape mechanisms. The next step would then be to wait until resistant subpopulations would present themselves and then rescreen these tumors against RNAi libraries to identify key genes to such resistance mechanisms, targeting them in the effort to induce complete remission.

6.3 The regulation of the PI3K and the MAPK pathways in IGF-1R dependent cells

Gotoh 2008 (234) showed how IRs and IGFRs would usually induce a more modest activation of the MAPK pathway compared to FGFRs. In this thesis I support this observation showing how in bladder cancer cells, or at least in the IGF-1R dependent cells, the IGF-1 receptor is a much better regulator of the PI3K pathway than the MAPK pathway. Inhibition of this particular growth factor receptor results in decreased AKT phosphorylation, showing only little or no effects on ERK phosphorylation. This is consistent with data published, but not commented, by Herrera-Abreu *et al.*(200) showing how ERK phosphorylation in RT112 is indeed regulated by FGFR, while no effects are detected on AKT phosphorylation. Analyzing the data in Figure 5.1 in more detail, I realized how all the sensitive cell lines, whose IC50 is reached at submicromolar concentrations, are also dependent on either FGFR or EGFR. This singular observation captured our attention at the beginning of this study, pushing us in further investigating this double growth factor receptor dependency. Western blots on RT112, UC14 and UC6 show how in these sensitive cell lines the phosphorylation of AKT decrease in a time-dependent fashion when these cells are exposed to BMS-754807, but not to FGFR or EGFR

inhibitors. On the other hand FGFR seems to have effects only on the phosphorylation status of ERK1/2 but not on AKT in UC14 and RT112. Similar to the FGFR inhibitor, the effect of the EGFR inhibitor on UC6 cells showed no decrease in AKT phosphorylation, but complete depletion of P-ERK. It is still unclear to us whether this type of downstream target regulation is only happening in IGF-1 receptor dependent cells or it is something common to all bladder cancer cells. The results shown in figure 3.12 would suggest that at least in UC16 this is not happening.

Because of the double dependency of IGF-1Ri sensitive cells, I decided to test the effects of the combination of BMS-754807 with FGFR and EGFR inhibitors on proliferation. Interestingly, at least additive effect has been shown when the IGF-1R inhibitor is combined with a second growth receptor inhibitor that a certain cell is dependent on. To the contrary, when BMS-754807 is combined with FGFR inhibitor on EGFR dependent cells or EGFR inhibitor on FGFR dependent cells, the inhibitory effects of BMS-754807 are greatly attenuated and in certain cases and at certain concentrations, even reversed. These data would encourage the use of combinations of growth factor receptor inhibitors in clinic, but at the same time they should warn physicians of the possible negative effects of using these combinations without exactly knowing on which growth factor receptor tumors depend on. Another tempting targeted combination that has not been investigated in this thesis, is the possible combination of EGFR/FGFR inhibitors with PI3K pathway inhibitors like for example, the very effective double mTOR inhibitor AZD-8055. The advantage of this approach is that even in cell lines that are not dependent on IGF-1R, EGFR and FGFR are not able to effectively modulate the PI3K pathway (data not shown). The combination of AZD8055 with FGFR inhibitors like AZD-4547 or BGJ398 or with EGFR

inhibitors like Iressa, would allow us to obtain a more beneficial effect in a larger subset of bladder cancers.

It is intriguing to wonder on what kind of mutations or epigenetic changes are the cause of this phenomenon of double-dependency or alternatively speculate whether this phenomenon is already present in normal urothelial cells but lost in most cancer cells. Further and deeper studies would be necessary to address this issue. It is tempting to speculate that alteration of adaptor proteins could be a potential answer, as TCGA data from Figure 6.1 show how in bladder cancer alterations in growth factor receptors' adaptor proteins have been detected in 100% of the cases.

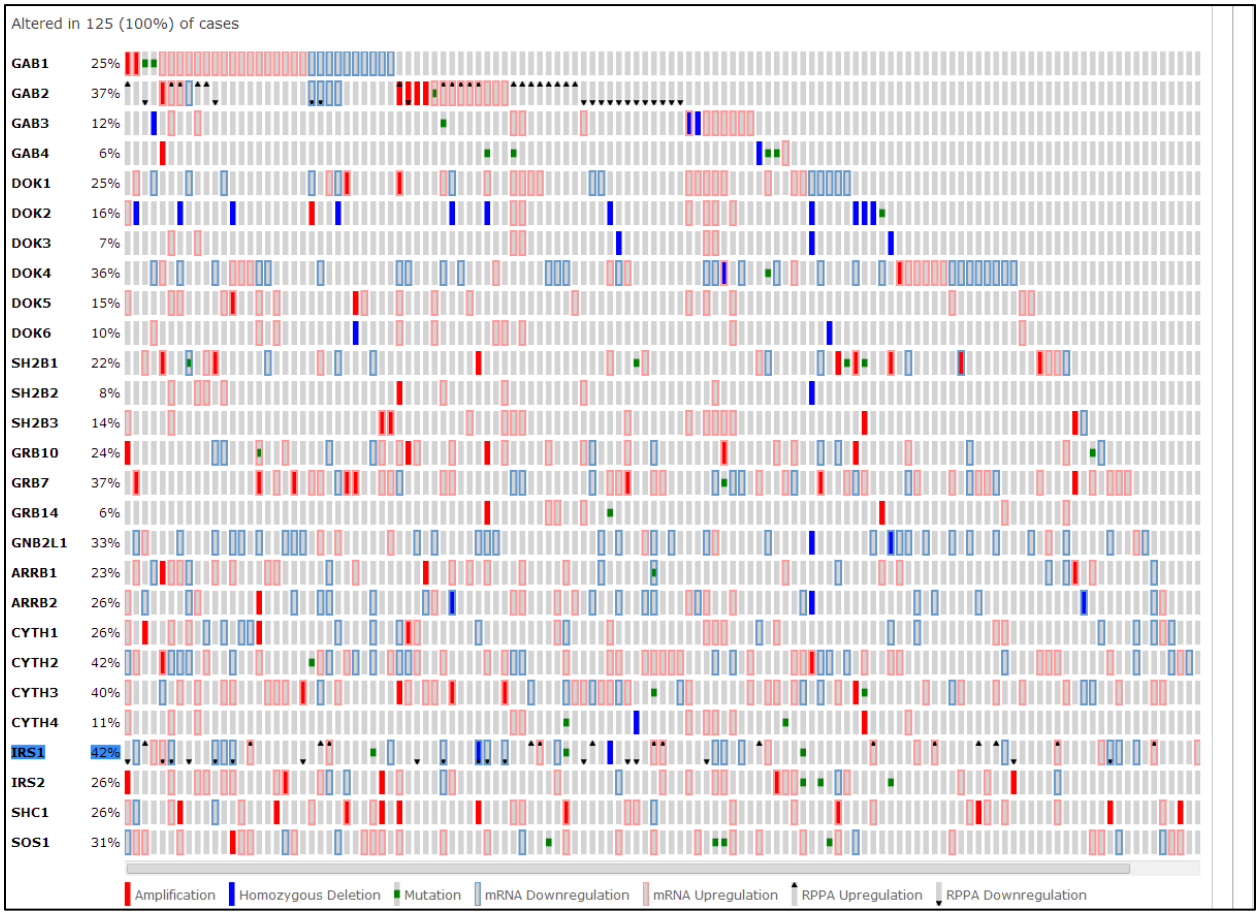

Figure 6.1: TCGA data show the pattern of adaptor proteins alteration in bladder cancer patients. Generated by using the software provided at this url: http://www.cbioportal.org/public-portal/

CHAPTER 7. REFERENCES

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VITA

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